

**FORMATION OF ELECTROSTATIC COMPLEXES WITHIN ADMIXTURES OF
LENTIL PROTEIN ISOLATES AND ANIONIC POLYSACCHARIDES
(κ -CARRAGEENAN, DE-ACYL GELLAN GUM AND GUM ARABIC)**

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ABSTRACT

Proteins from plant sources are generally less soluble and have poorer functionality compared to animal proteins. The overall goal of this thesis was to better understand mechanisms associated with the formation of electrostatic complexes involving mixed systems of lentil protein isolates (LPI) and three different anionic polysaccharides (gum Arabic (GA), κ -carrageenan (κ -CG) and de-acyl gellan gum (GG)). A better understanding of mixed systems should lead to the development of formulated ingredients for targeted applications. Findings also may lead to enhanced utilization of lentil proteins as food and/or biomaterial ingredients with improved functionality over the protein alone.

Maximum complexation occurred in the 1:1 LPI:GA mixed system (total biopolymer concentration (C_p) = 0.05%, w/w) at pH 3.50 with complexation following two pH-dependent structure forming events associated with the formation of soluble (pH_c) and insoluble ($pH_{\phi 1}$) complexes at pH 5.87 and 3.62, respectively. The addition of GA resulted in a shift of the LPI isoelectric point (pH 4.70) to a lower pH (3.17). The addition of sodium chloride (NaCl) disrupted coacervation, whereas the addition of urea caused a drop in the magnitude of the observed maximum optical density (O.D.). Increasing the temperature to 60°C resulted in a shift in turbidity curves towards more acidic pH and a decrease in maximum O.D. relative to the control (21-23°C).

The addition of GG or κ -CG to LPI resulted in a suppression of LPI aggregation by electrostatic repulsion with a shift in net neutrality of the formed complexes to a lower pH (4.36) compared to LPI alone (pH 4.70) as measured by electrophoretic mobility of a 15:1 LPI:GG/ κ -CG mixed system (C_p = 0.05%, w/w). The addition of salts resulted in disruption of formed LPI:GG/ κ -CG complexes, and no polysaccharide-ion specific sensitivities were evident (i.e., Ca^{2+} to GG or K^+ to κ -CG).

Complexation was primarily driven by electrostatic attractive forces with secondary stabilization by hydrogen bonding. Hydrophobic interactions were thought to play a role in the stabilization of LPI-LPI aggregates. Removal of the lentil hull had a minor effect on complexation. Initial interactions occurred slightly above the pI of the LPI where biopolymers carried net negative charges with polysaccharide chains interacting with positive patches on the protein's surface.

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LIST OF SYMBOLS AND ABBREVIATIONS

LPI	Lentil protein isolate
GA	Gum Arabic
GG	De-acyl gellan gum
κ -CG	κ -Carrageenan
GDL	Glucono-delta lactone
g	gravitational force
pI	Isoelectric point
Cp	Total biopolymer concentration
K_a	Acid dissociation constant
p K_a	$-\log K_a$
kDa	Kilo Daltons
O.D.	Optical density
pH	$-\log [H^+]$
pH _c	Critical pH at which soluble complexes form
pH _{ϕ_1}	Critical pH at which insoluble complexes form
pH _{opt}	pH where maximum coacervate formation occurs
pH _{ϕ_2}	Critical pH at which complexes dissociate
rpm	Rotations per minute
min	Minutes
°C	Degrees Celsius
α	Alpha
β	Beta
κ	Kappa
λ	Lambda
ι	Iota
mm	Millimetre
nm	Nanometre
mM	Millimolar
M	Molar

mV	Millivolts
dL	Deciliter
S	Svedberg unit

1. INTRODUCTION

1.1 Summary

The overarching goal of this research was to better understand mechanisms associated with the formation of electrostatic complexes involving mixtures of lentil protein isolates and a variety of anionic polysaccharides (κ -carrageenan (κ -CG), de-acyl gellan gum (GG) and gum Arabic (GA)). The protein ingredient industry is currently dominated by animal derived proteins, such as gelatin, whey, casein and ovalbumin. However the demand for plant-based alternatives is on the rise in the food industry due to consumer perceived fears surrounding diseases (e.g., prion), dietary preferences and food choices based on moral, religious and cultural prohibitions that restrict the use of animal derived products (Liu et al., 2009; Boye et al., 2010a). Unfortunately plant proteins tend to have poorer functional properties than the animal proteins, especially as it relates to protein solubility. Controlled protein-polysaccharide interactions through complex coacervation may offer a means for improving their performance as ingredients without chemical or enzymatic modification (Schmitt et al., 1998; Weinbreck et al., 2004a; Liu et al., 2009). Findings from this work will help form the foundation for designing new formulated plant protein-based food and/or biomaterial ingredients for a host of applications that could have improved functionality over the protein alone.

Complex coacervation (also known as associative phase separation) involves the separation of a colloidal system into two liquid phases: biopolymer- and solvent-rich phases and it typically involves the electrostatic attraction of opposing charged biopolymers, such as an anionic polysaccharide and a positively charged protein at pHs below its isoelectric point (de Kruif et al., 2004). Protein-polysaccharide interactions tend to be inherently electrostatic in nature; however the secondary role of other non-specific interactions is less well understood (Schmitt et al., 1998; de Kruif and Tuinier, 2001). Complexation studies involving plant proteins have been limited especially as they relate to mechanisms of interaction and their assembly into higher ordered structures, and the influence of physicochemical factors (pH, ionic strength, mixing ratio, polysaccharide (reactive group)-type, and molecular weight) on complex

formation/stability, aggregate growth and functionality. The present study investigates the effect of solvent (pH and salt) and biopolymer characteristics (polysaccharide-type, total biopolymer concentration and biopolymer mixing ratio) on mechanisms governing complex coacervation involving lentil protein isolates and anionic polysaccharides (κ -carrageenan, de-acyl gellan gum and gum Arabic). Lentil (*Lens culinaris*) protein was chosen to study since it is currently not utilized (as a protein ingredient), it is nutritious and, is an economically important crop for Saskatchewan and the global legume market (Agriculture and Agri-Food Canada, 2010).

1.2 Objectives

The objectives of the thesis are as follows :

- a. identifying optimal conditions for complex coacervation between lentil protein isolates (LPI) with κ -CG, GG and GA polysaccharides;
- b. investigating the nature of interactions involved with the complexation of LPI and GA;
- c. investigating the effect of dehulling (or removal of the seed coat) on complex formation involving LPI and GA; and
- d. examining the effect of monovalent and divalent ions on the stability of electrostatic complexes involving LPI with κ -CG and GG.

1.3 Hypotheses:

The following hypotheses were tested within the proposed research, including:

- a. LPI:GA interactions will lead to the formation of a coacervates, whereas interactions with more highly charged polysaccharides (κ -CG and GG) will lead to precipitation.
- b. Optimal conditions for LPI-polysaccharide complex formation will occur at $\text{pH} < \text{pI}$ of LPI.
- c. Complexation of LPI and polysaccharides will mainly be driven by electrostatic attractive forces with secondary stabiliztion by hydrogen bonding.
- d. The presence of the hull will have little effect on complexation between biopolymers.
- e. The addition of K^+ and Ca^{2+} ions will lead to an initial increase in complex formation, however at higher levels segregative phase separation will ensue.

2. LITERATURE REVIEW

2.1 Introduction

Currently, the global protein ingredient market is dominated by animal-derived proteins such as casein, whey, ovalbumin and gelatin, with soy dominating the plant-based protein ingredient market. However, with increased perceived consumer fears of animal-based products (e.g., prion diseases), changing dietary preferences and diet restrictions because of religious or moral beliefs, the food industry is searching for plant-based alternatives (Liu et al., 2009). Furthermore, consumers are also concerned with soy-based products associated with allergies, flavor and flatulence. Potential alternatives could include proteins arising from legume or oilseed crops; however the functionality attributes tend to be poorer than those of the current proteins on the market. For many decades, researchers have been trying to enhance the functionality of plant proteins through chemical (Matsudomi et al., 1985; Ponall et al., 2010) or enzymatic (Hettiarachchy et al., 1985; Ortiz and Wagner, 2002) means with only some success. Alternatively, another approach could involve controlling protein-polysaccharide interactions to coat the surface of the protein to change its physicochemical properties and then ultimately its functional behavior. Proteins and polysaccharides are macromolecules commonly used by the food industry in a wide range of applications due to their emulsifying, foaming, thickening or gelling properties. Knowledge of structure-dynamic-function relationships relating to their interactions in food systems is especially important as it often controls food texture, structure, processability and shelf life (Dickinson and Pawlowsky, 1998; Schmitt et al., 1998; Doublier et al., 2000; Weinbreck et al., 2004a; Mounsey et al., 2008; Liu et al., 2010a,b). Protein-polysaccharide interactions can also be tailored for the design of controlled delivery vehicles for carrying bioactive ingredients, or for developing coating and packaging materials (Liu et al., 2009). Depending on the nature of interactions and the biopolymers involved, formed electrostatic complexes (coacervates or precipitates) may have improved functionality than the biopolymers alone (Tolstoguzov, 1991; Schmitt et al., 1998).

Although the mechanism for complex coacervation has not been fully elucidated, the topic has been reviewed by Turgeon et al. (2007). Most studies have previously focused on complex coacervation between animal-derived proteins, such as bovine serum albumin (Wang et al., 1996; Wen and Dubin, 1997), casein (Syrbe et al., 1998), whey proteins (Weinbreck et al., 2003a,b, 2004a,b) and β -lactoglobulin (Schmitt et al., 1999; Harnsilawat et al., 2006), and anionic polysaccharides. In contrast, mechanistic studies of coacervation involving plant proteins have been limited. Liu et al. (2009, 2010a,b) investigated the effect of pH, salt and biopolymer mixing ratio on complex formation involving pea protein-GA mixtures, and characterized both the nature of interactions and the functional attributes of formed complexes. Similar mechanistic studies were also performed for mixtures of canola protein- ι -carrageenan/alginate (Klassen et al., 2011), pea protein-alginate (Klemmer et al., 2012), pea protein-chitosan (Elmer et al., 2011), and pea globulin-GA (Ducel et al., 2004; Chourpa et al., 2006).

The present study investigates the effect of solvent (pH and salt) and biopolymer characteristics (polysaccharide-type, total biopolymer concentration and biopolymer mixing ratio) on mechanisms governing complex coacervation involving lentil protein isolates and anionic polysaccharides (κ -CG, GG and GA). Knowledge of these mechanisms could lead to the development of ‘formulated protein-polysaccharide ingredients’ with unique functionality. Lentil proteins were selected for this study since it is currently not utilized as a protein source for food ingredient purposes and is nutritious (i.e. lentils contain - ~ 25% protein, 56% carbohydrate, and 1% fat and is an excellent source of minerals, such as Ca^{2+} , Fe^{2+} , Na^+ , Mg^{2+} and K^+ (Swanson, 1990; Boye et al., 2010a; Ahmed, 2010)); and is an economically important legume crop for Saskatchewan and Canada (Agriculture and Agri-Food Canada, 2010). In the case of the selected anionic polysaccharides: κ -CG is a linear sulphated polysaccharide that is sensitive to K^+ salts; GG is a linear carboxylated polysaccharide that is sensitive to Ca^{2+} salts; and GA is a branched carboxylated polysaccharide. Mixtures involving these polysaccharides will enable exploration of the role ion sensitivity and strength of reactive sites play during complex coacervation with plant proteins.

2.2 Coacervation

In very dilute biopolymer mixtures, proteins and polysaccharides remain co-soluble (Fig. 2.1) with limited interactions within the aqueous solution (Weinbreck et al., 2003a; Ye, 2008).

However, as the total biopolymer concentration is raised either segregative- or associative- type phase separation (Fig. 2.1) may occur depending on the solvent conditions (i.e., temperature, pH and salts), biopolymer characteristics (i.e., polysaccharide-type, reactive site, molecular weight, linear charged density and conformation), biopolymer concentration and mixing ratio, and physical processing (i.e., pressure, degree and time of shear) (Tolstoguzov, 2003; Gharsallaoui et al., 2010; Liu et al., 2009, 2010a). Segregative phase separation occurs when the two biopolymers are thermodynamically incompatible, typically driven by electrostatic repulsive forces between proteins and polysaccharides of similar net charges. Separation leads to both a protein-rich and polysaccharide-rich phase (Doublier et al., 2000; Weinbreck et al., 2003a,b; Liu et al., 2009, 2010a). In contrast, associative phase separation (also known as complex coacervation) occurs when the biopolymers carry opposite net charges and experience electrostatic attractive forces (Bungenberg and Kruyt, 1929). This charge interaction leads to separation into a biopolymer-rich (proteins + polysaccharides) and a solvent-rich phase, and typically occurs over a narrow pH range between the pK_a of the reactive site on the polysaccharide backbone and the isoelectric point (pI) of the protein (Doublier et al., 2000; Weinbreck et al., 2003a,b; Liu et al., 2009, 2010a). The formed ‘electrostatic complexes’ can either be present as a coacervate or a precipitate depending on the level of electrostatic attraction.

In either case, structure formation tends to follow a similar nucleation growth-type kinetic mechanism involving two main pH-dependent structure-forming events (Girard et al., 2004; Liu et al., 2009). During an acid titration involving a mixture of proteins and an anionic polysaccharide, structure formation is typically accessed by changes to turbidity (de Kruif and Tuinier, 2001; Turgeon et al., 2003; Weinbreck et al., 2003a; Liu et al., 2009) or scattering intensity (Semenova, 1996; Girard et al., 2004; Harnsilawat et al., 2006). The first experimentally detected rise in turbidity signifies the onset of structure formation in the form of soluble complexes, occurring at a pH denoted as pH_c (Fig. 2.2). As the pH is lowered further, macroscopic phase separation occurs as evident by a large rise in turbidity and a transition from a clear to cloudy solution (Weinbreck et al., 2003a; Liu et al., 2009). The onset of this rise is denoted as $pH_{\phi 1}$ (Fig. 2.2) and corresponds to the formation of insoluble complexes. The terms ‘soluble’ and ‘insoluble’ are terms used in the coacervation literature to describe the various stages of growth and do not relate to ‘solubility-like’ functional testing. As the pH is lowered further, the mixture reaches its electrical equivalent point and a maximum in turbidity or scattering intensity occurs.

At this pH (denoted as pH_{opt}) (Fig. 2.2), electrostatic complexes become neutral and yield the greatest concentration of formed structures (Weinbreck et al., 2003a; Liu et al., 2009). At lower pH, the polysaccharide becomes protonated and previously formed structures begin to dissociate until reaching $\text{pH}_{\phi 2}$ (Fig. 2.2), where complete dissolution of complexes occurs (Weinbreck et al., 2003a; Liu et al., 2009).

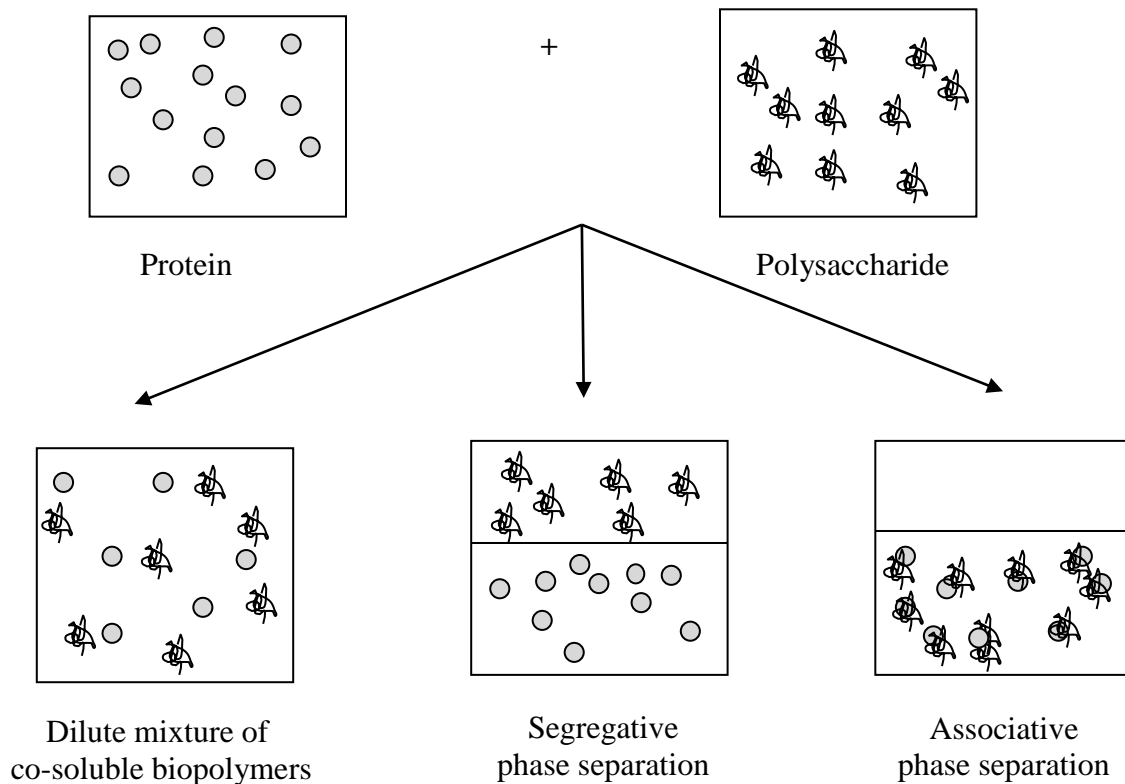


Figure 2.1 A schematic outlining segregative and associative phase behavior in admixtures of protein-polysaccharide systems (adapted from de Kruif and Tuinier, 2001).

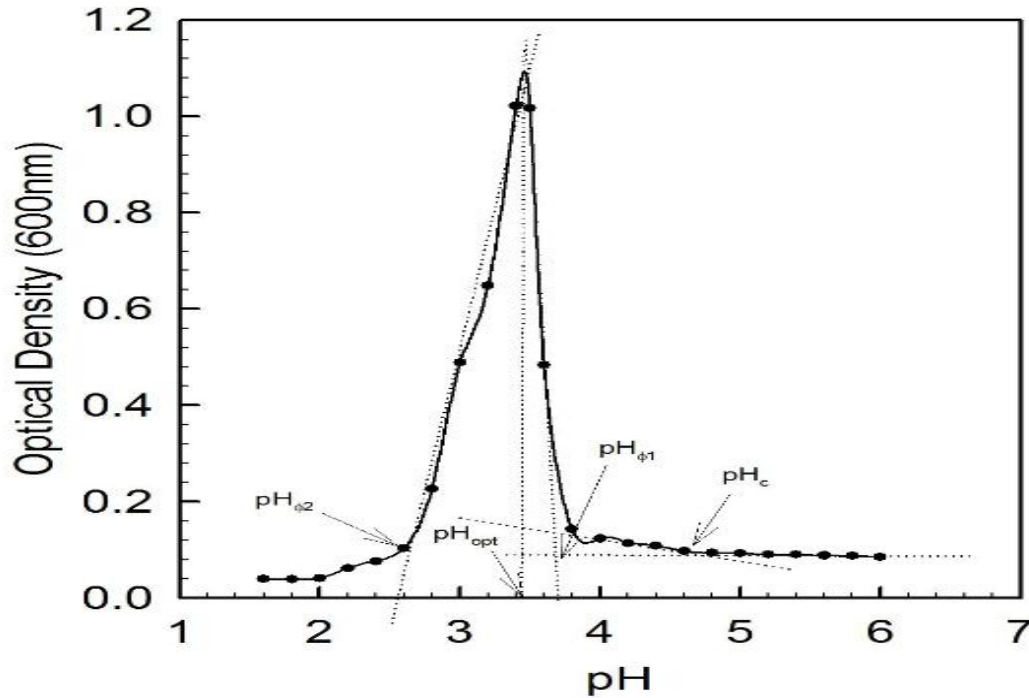


Figure 2.2 Schematic diagram showing how critical transition pHs (pH_c , $\text{pH}_{\phi 1}$, pH_{opt} and $\text{pH}_{\phi 2}$) are determined from a turbidity curve (Liu et al., 2009).

The coacervate structure is reversible, entraps a sufficient amount of solvent to remain suspended, and typically occurs within mixtures of proteins and weakly charged polysaccharides (i.e., those having a relative low linear charge density or weakly charge reactive group, such as the carboxyl sites found on GA and guar gum) (de Kruif et al., 2004). A stable coacervate structure is formed as entropies associated with biopolymer flexibility and solvent mixing become reduced, which offsets the enthalpic contribution associated with the release of water and counterions during complex formation (Schmitt et al., 1999; Singh et al., 2007; Ye, 2008). In contrast, the precipitate structure tends to be only partially reversible and falls out of solution quickly. Biopolymers within the precipitate are more compact and entrap a lesser amount of solvent than the coacervate structure (Boral and Bohidar, 2010). In general, precipitation occurs within mixtures of proteins and strongly charged polysaccharides (i.e., those having a relatively high linear charge density of highly charge reactive group, such as carrageenan (sulfate group), chitosan (amine group) or exocellular polysaccharide B40 (phosphate group)) (Schmitt et al., 1998; Weinbreck et al., 2003b; de Kruif et al., 2004). Although complex coacervation is primarily driven by electrostatic attractive forces, the role of secondary forces, such as hydrophobic interactions and hydrogen bonding is less understood (Bungenberg and Kruyt,

1929; Schmitt et al., 1998; Doublier et al., 2000; Kaibara et al., 2000; de Vries et al., 2003; Weinbreck et al., 2003b; Wang et al., 2007; Liu et al., 2010a). Liu et al. (2010a) studied the nature of interactions within a mixture of pea protein isolate and gum Arabic and found that coacervation was governed primarily by electrostatic forces, with secondary stabilization by hydrogen bonding. The authors found that hydrophobic interactions played a role in stabilizing the protein-protein aggregates bound to the gum Arabic molecule, and reported that they were not involved in initial complex formation. However, in systems involving uncharged protein-polysaccharide mixtures, hydrophobic interactions have been reported to be the dominant driver for complex formation (Jönsson et al., 2003).

2.3 Factors affecting complex coacervation

Because complex coacervation involving proteins and charged polysaccharides is widely considered to be driven by electrostatic attractive forces, factors such as solvent pH, the presence of salts, biopolymer mixing ratio and polysaccharide-type are key to better understanding structure formation (Weinbreck et al., 2004a,b; Sanchez et al., 2006; Wang et al., 2007). The effect of each of these factors is briefly described below.

2.3.1 Effect of pH

Solvent pH is critical for altering the charge density on the protein's surface, which is required to trigger complexation with a charged polysaccharide (Schmitt et al., 1998; Jönsson et al., 2003; Weinbreck et al., 2004a,b; de Kruif et al., 2004). At $\text{pH} > \text{pI}$ conditions, the protein assumes a net negative charge, whereas at $\text{pH} < \text{pI}$ a positive net charge is introduced. Complex coacervation typically occurs within a narrow pH range between the pK_a of reactive groups along the polysaccharide backbone and the pI of the protein (Weinbreck et al., 2003b). As previously mentioned, as pH is lowered to $\text{pH} < \text{pI}$ the protein assumes a positive net charge leading to the formation of an initial soluble complex at pH_c , by interacting with the negatively charged polysaccharide. In systems involving highly charged polysaccharides, such as carrageenan, pH_c can occur at $\text{pH} > \text{pI}$ where both biopolymers carry a similar net positive charge (Weinbreck et al., 2003a, 2004a; Liu et al., 2009). Typically this phenomenon is thought to be associated with the interaction between the highly charged polysaccharide with positively charged patches of amino acids on the protein's surface (Weinbreck et al., 2003a, 2004a; Liu et

al., 2009). As the pH is lowered during the acid titration, structure growth leads to the formation of insoluble complexes at $\text{pH}_{\phi 1}$, and then reaches pH conditions where charges along the protein and polysaccharide's surface are equivalent (pH_{opt}). At lower pH conditions, the reactive sites along the polysaccharide backbone become protonated leading to a weakening of the attractive forces between biopolymers until reaching $\text{pH}_{\phi 2}$ where dissolution of complexes occurs (Schmitt et al., 1998; Jönsson et al., 2003; de Kruif et al., 2004; Weinbreck et al., 2004a,b).

2.3.2 Effect of ionic strength

The presence of cations and anions has a dramatic effect on complex formation and their role is concentration dependent. As examples, in whey protein isolate-GA mixtures, NaCl levels >50 mM acted to suppress coacervation due to screening of the electric double layer on both the protein and the polysaccharide by bound and unbound ions (Weinbreck et al., 2003a). Liu et al. (2009) reported that critical pHs associated with complexation within pea protein isolate-GA mixtures were difficult to assess at levels >7.5 mM NaCl due to the increased prevalence of pea protein aggregates in the system. When investigating the nature of interactions involved with complexation of the same system, the authors found that complexation to be completely suppressed at 100 mM NaCl levels (Liu et al., 2010a). A number of research groups have reported similar findings, where the complexation of β -lactoglobulin-(high and low methoxy) pectin (Girard et al., 2002), canola protein isolate-(ι -carrageenan/alginate) (Klassen et al., 2011) and whey protein isolate-exocellular polysaccharide EPS B40 (Weinbreck et al., 2003a) mixtures were suppressed at 110, 100 and 75 mM NaCl respectively.

In contrast, in the presence of low salt concentration, complexation may be enhanced. As examples, Weinbreck et al. (2003a) reported that complexation within whey protein isolate-GA mixtures were improved at NaCl levels <50 mM. Under these ionic conditions, biopolymer-ion interactions may lead to slight conformational changes to the protein structure to expose or partially expose reactive sites and improve biopolymer flexibility within solution (Weinbreck et al., 2003a, Duce et al., 2004). Low levels of NaCl may also play a role in enhancing protein solubility, which in turn will lead to greater interactions with polysaccharides in solution. The influence of NaCl addition has been well studied in literature for mixtures of pea protein isolate-GA (Liu et al., 2009, 2010a), β -lactoglobulin-pectin (Girard et al., 2004) and whey protein isolate-GA (Weinbreck et al., 2003a). Weinbreck et al. (2004a) reported for a whey protein

isolate-carrageenan mixture that in the presence of NaCl, $pH_{\phi 1}$ is shifted to higher pH as NaCl levels increased up to 45 mM. At levels of 1.0 M NaCl, complexation of the same system was suppressed, with the higher amount of NaCl needed to suppress complexation attributed to the greater strength of the sulphated carrageenan versus the weaker carboxylated polysaccharides. The influence of others salts on complexation is less understood and studied. Weinbreck et al. (2004a) studied the effect of $CaCl_2$ addition on complex formation ($pH_{\phi 1}$) involving whey protein isolate and λ -type carrageenan mixtures. The authors reported a pH shift associated with complex formation to higher pH (8.00), which the authors attributed to calcium bridging.

2.3.3 Effect of biopolymer mixing ratio and total biopolymer concentration

Biopolymer mixing ratio also has a significant effect on complex formation, as it influences the level of available sites for electrostatic attraction between the two biopolymers. In general, the formation of soluble complexes is considered to be unaffected by mixing ratio, where it is thought that complexation involves the interaction between a single polysaccharide chain with a few protein molecule (Weinbreck et al., 2003a). However, this hypothesis was primarily shaped from studies involving ‘aggregate-free’ whey protein-polysaccharide mixtures (Weinbreck et al., 2003a,b, 2004a,b). In contrast, Klassen et al. (2011) and Liu et al. (2009, 2010a,b) reported for canola protein isolate (ι -carrageenan/alginate) and pea protein isolate-GA mixtures, respectively, that pH_c was dependent on the mixing ratio. The authors proposed that the mixing ratio dependence arose because the proteins were strongly aggregated, and initial complexes formed between small protein-protein aggregates with a single polysaccharide chain. The biopolymer mixing ratio dependence reflects the progressive growth of protein–protein aggregates with increasing biopolymer concentration until a critical size is reached, after which a steady state occurs. Similar biopolymer mixing ratio dependence for pH_c was reported by Singh et al. (2007) involving mixtures of agar with type-A and type-B gelatin. In contrast, it is generally accepted that there is a mixing ratio dependence associated with the formation of insoluble complexes, where $pH_{\phi 1}$ increases with mixing ratio up to a critical value before reaching a steady state. This trend is believed to be attributed to a greater amount of protein molecules available per polysaccharide chain for binding (Weinbreck et al., 2003a,b, 2004a,b; Liu et al., 2009; Klassen et al., 2011). As an example, Weinbreck et al. (2003b) reported a shift in $pH_{\phi 1}$ to higher pH values as mixing ratios increased from 1:1 to 9:1 in whey protein isolate–

exocellular polysaccharide B40 mixtures, above which charges along the protein's surface became saturated. A similar trend was reported for whey protein-carrageenan and pea protein isolate-GA mixtures where protein saturation occurred at mixing ratios of 30:1 and 4:1, respectively (Weinbreck et al., 2004a; Liu et al., 2009).

Typically, coacervation studies are prepared under very dilute biopolymer conditions (0.05-0.1%, w/w), since above a critical total concentration, coacervation of biopolymer is suppressed and interactions are more difficult to access via scattering techniques (e.g., turbidity). Suppression at higher total biopolymer concentrations arise due to competition for available solvent molecules between the two biopolymers, and an increased release of counterions into solution which screen reactive sites along the biopolymer's electric double layer (Weinbreck et al., 2003a; Ye, 2008; Liu et al., 2009). Liu et al. (2010b) studied the effect of pH on the functional attributes of pea protein isolate-GA complexes at high total biopolymer concentrations (5% w/w) and found that biopolymer complexation was still occurring.

2.3.4 Effect of polysaccharide-type

de Kruif et al. (2004) classified polysaccharides used in coacervation studies as being either weakly or strongly charged. Polysaccharides that have a relatively low charge density and weak reactive site are considered weakly charged and typically lead to the formation of coacervate-type structures. These polysaccharides include: GA, pectin, guar gum, carboxyl methyl cellulose and xanthan gum. In contrast, strongly charged polysaccharides have a high linear charge density and/or a highly charged reactive site (e.g., sulfate, amine or phosphate group). These polysaccharides could include: carrageenan, chitosan alginate, GG, and exocellular polysaccharide B40.

Dickenson (1998) and Doublier et al. (2000) reported that $-\text{OSO}_3^-$ groups had greater attraction to $-\text{N}^+\text{R}_3$ groups on the protein's surface than $-\text{COO}^-$ groups. Depending on the polysaccharide-type, the reactive groups can differ resulting in differences in chain flexibility (or conformational entropy). Polysaccharides can also be linear in nature or branched; potentially affecting interactions with proteins due to steric hindrance. As previously mentioned, complex formation involving proteins and weakly charged polysaccharides typically occurs at $\text{pH} < \text{pI}$ conditions, however in the presence of highly charged polysaccharides complexation can be initiated at $\text{pH} > \text{pI}$ due to interactions with positively charged patches on the protein's surface.

2.3.5 Effect of molecular weight

The coacervation process has been predicted to be influenced by the molecular weight of the biopolymers within the mixed system where an increase in biopolymer molecular weight leads to an increase in the degree of coacervation (Overbeek and Voorn, 1957). Studies on the coacervation of soy globulin and dextran using light scattering have shown that the level of coacervation increased with increasing molecular weight of the dextran molecules (Semenova, 1996). The increased volume occupied by larger biopolymers is presumed to enable greater access to reactive sites for other macromolecules to react with during the coacervation process (Semenova, 1996; Schmitt et al., 1998). However, this trend is not always followed. Shieh and Glatz (1994) studied the complexation between lysozyme and polyacrylic acid (PAA) at different molecular weights ranging in size from 5 kDa to 4,000 kDa to find molecular weight played only a minor role in complex formation.

2.3.6 Effect of processing conditions

Complex formation and stability have been shown to be influenced by processing conditions, such as temperature, shearing and pressure (Schmitt et al., 1998). These processing conditions can influence the formation and/or stability of coacervates by partial or complete denaturation of proteins and hence influence their conformation by exposing buried hydrophobic groups which will favour non-polar interactions. High or low temperatures also break or favour hydrogen bonding (Schmitt et al., 1998). These processing conditions may induce protein-protein aggregation, effectively increasing the molecular weight of the interacting species. Post-harvest processing of plant materials prior to protein extraction could also impact behavior and hence complexation.

2.3.7 Role of protein aggregation

Protein-protein aggregation influences complexation during an acid titration by: i) limiting the available reactive sites on the protein's surface for complex formation, ii) forming larger aggregates for interactions with polysaccharides and, iii) altering the biopolymer mixing ratio required to reach neutrality and maximum yield (Schmitt et al., 2000, 2001; Sanchez and Renard, 2002). Protein-protein aggregates have been reported to play a stabilizing role in β -lactoglobulin-acacia gum (Schmitt et al., 2000, 2001; Sanchez and Renard, 2002), pea protein

isolate-GA systems (Liu et al., 2009, 2010a) due to protein aggregates size distribution and stabilizing hydrophobic interactions within the aggregated structure relative to aggregate-free systems; whey protein isolate-GA (Weinbreck et al., 2003a). Approximately 60-70% of the acacia gum has been reported to react to form aggregates and precipitates in a β -lactoglobulin-acacia gum system (Schmitt et al., 2001). In contrast, aggregate-free solutions formed complexes via electrostatic attraction only where polysaccharide chains interacted with only a few protein molecules to form complexes (Weinbreck et al., 2003a). Protein-polysaccharide ingredients that stay stable without the need for chemical crosslinkers could be formulated if the role that protein aggregates play in the stabilization of coacervates is better understood.

2.4 Choice of biopolymers

2.4.1 Lentil protein isolate (LPI)

Lentil (*Lens culinaris*) proteins were selected for this research due to their nutritional value, functional properties and the economic importance of this crop to Saskatchewan and Canada. Lentils are primarily exported as whole seeds from Canada, however post-processing into protein isolates could add significant economic value. Currently, soy and pea protein ingredients are the only legume-based ingredients on the market. From a nutritional perspective, legumes are of particular interest because they contain high amounts of protein (18–32%) and contain significant amounts of minerals, essential amino acids and bioactive peptides (Boye et al., 2010a). Legume proteins also possess functional properties such as water holding, fat binding, foaming and gelation which could expand their use for the development of a wide variety of food products (Boye et al., 2010a). Lentil proteins are comprised mainly of globulins and albumins, with the former occurring in greater quantities (Swanson, 1990; Neves and Lourenco 1995; Boye et al., 2010a). Bhatti (1988) showed that albumins and globulins represented 19.6 and 53.9% of the solubilized lentil protein, respectively. Globulins can be divided into two main types based on their sedimentation coefficients of 7S (~150 kDa) (S, Svedberg Unit) and 11S (~350-400 kDa), referred to as vicilin and legumin-like proteins because of analogy to vicilin and legumin from pea seeds. In lentils, the 11S globulin represents the major fraction (Swanson, 1990; Neves and Lourenco 1995; Lampart-Szczapa, 2001; Boye et al., 2010a).

Recent research studies have suggested that whole legume consumption may have potential health benefits including reduced risk of cardiovascular disease, cancer, diabetes, osteoporosis, hypertension, gastrointestinal disorders, adrenal disease and the reduction of low density lipoprotein cholesterol (Hu, 2003; Tharanathan and Mahadevamma, 2003). Green lentils also have green seed coat (hull) with yellow cotyledons. Typically, lentils are utilized for human consumption in soups, stews, salads, as meat extenders, and gluten-free diets once the seed coat has been removed. The hull or seed coat is often indigestible and is bitter tasting. Removal of the seed coat helps improve palatability and taste, and as such plays an important role in processing and utilization of lentils (Wang, 2005). Wang (2005) observed that hulls contain indigestible fibre, polyphenols, tannins and other anti-nutritional factors which impart a bitter taste and may also impact flour/protein physicochemical properties. To the best of our knowledge there is nothing in literature about the effect the content of the seed coat on the complexation with polysaccharides.

2.4.2 κ -Carrageenan (κ -CG)

κ -Carrageenan is a water-soluble linear anionic sulfated polysaccharide extracted from red seaweed (*Rhodophyceae*), used primarily as a gelling agent in a wide range of food applications (Clark and Ross-Murphy, 1987; Chandrasekaran and Radha, 1995; Ould et al., 2000a,b; Nickerson and Paulson, 2004; Rodrigues et al., 2012). κ -Carrageenan consists of repeating disaccharide units of alternating (1 \rightarrow 3)- α -D-galactopyranose-4-sulphate and (1 \rightarrow 4)- β -3,6-anhydro-D-galactopyranose (Fig. 2.3) monomers (Harding et al., 1997, Ueda et al., 1998; Ould et al., 2000a,b; Nickerson and Paulson, 2004; Rodrigues et al., 2012). κ -Carrageenan typically forms a gel on cooling a hot sol (liquid dispersion containing particles of colloidal dimension); occurring in a two-step process, characterized by a coil-helix transition followed by aggregation and network formation (Morris, et al., 1980; Rochas and Rinaudo, 1980; Hermansson et al., 1991). The gelling behavior is highly influenced by the type and concentration of cations present in solution, as well as the polymer concentration and cooling rates (Rochas and Rinaudo, 1980; Hermansson et al., 1991; Baeza et al., 2002). In the presence of K^+ , ion-mediated junction zones lead to the formation of multi-stacked single or double helices. κ -Carrageenan's sensitivity to K^+ leads to increased gel strength relative to other ions (e.g. Ca^{2+}). As such, the effects of κ -CG sensitivity to K^+ on coacervation was explored in this

research. Similar to other polysaccharides, pH has little or no effect on the gel transition temperature of κ -CG and only marginal effects on gel strength (Singh and Jacobsson 1994; Drohan, et al., 1997; Mleko, et al., 1997; Hermansson et al., 1991; Nickerson and Paulson, 2004; Nickerson et al., 2004). Previously, complex coacervation studies have been investigated involving κ -CG with proteins such as, casein (Bourriot et al., 1999), whey protein (Weinbreck et al., 2004a) and soy protein isolates (Ortiz et al., 2004). In all cases, protein- κ -CG tends to lead to the formation of precipitate structures.

2.4.3 De-acyl gellan gum (GG)

De-acyl gellan gum is a water soluble linear microbial exocellular polysaccharide, secreted from *Sphingomonas elodea* (formerly classified as *Pseudomonas edodea*), and consists of repeating tetrasaccharide units of $[\rightarrow 3)\text{-}\beta\text{-D-glucopyranosyl-1}\rightarrow 4)\text{-}\beta\text{-D-glucuronopyranosyl acid-(1}\rightarrow 4)\text{-}\beta\text{-D-glucopyranosyl-(1}\rightarrow 4)\text{-}\alpha\text{-L-rhamnopyranosyl-(1}\rightarrow]_n$ (Fig. 2.4) (Chandrasekaran and Radha, 1995; Nickerson et al., 2003; de Jong and van de Velde, 2007). Gellan is sensitive to low concentrations of Ca^{2+} and forms Ca^{2+} -mediated junction zones by cross-linking two double helices (Chandrasekaran and Radha, 1995; Nickerson et al., 2003; Nickerson and Paulson, 2004). Similar to κ -CG, GG's sensitivity to Ca^{2+} leads to increased gel strength relative to other ions. Consequently, the effects of GG sensitivity to Ca^{2+} on coacervation was explored in this research. Because of its rheological properties, GG is used in a wide range of food products (such as puddings, dessert gels, frostings, beverages, dairy products, fruit spreads, bakery fillings, glazes, confections, icings, sauces, batters) and non-food applications (such as microbiological media, plant tissue culture media, room deodorant gels and controlled drug release), personal care products (such as sun screens, body lotions and mild hair conditioners, and clear gel toothpaste) (Nickerson et al., 2003; Nickerson and Paulson, 2004). To the best of our knowledge, complex coacervation studies involving gellan gum with proteins have not been reported in literature.

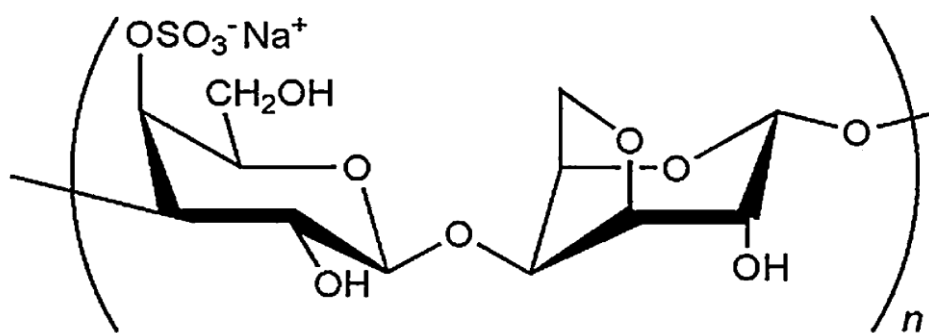


Figure 2.3 Disaccharide repeating unit of κ -carrageenan (adapted from Rodrigues et al., 2012).

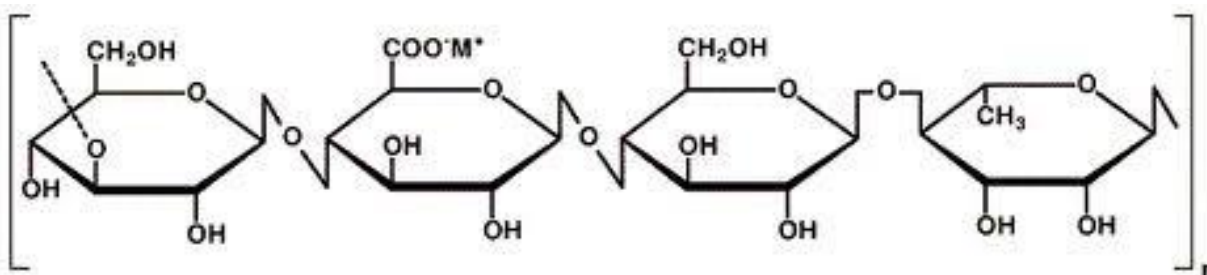


Figure 2.4 Tetrasaccharide repeating unit of de-acyl gellan gum (adapted from de Jong and van de Velde, 2007).

2.4.4 Gum Arabic (GA)

Gum Arabic is widely used by the food and pharmaceutical industries because it has a low viscosity at high concentrations and excellent emulsifying properties. It is a natural anionic plant polysaccharide extracted from *Acacia senegal* and *Acacia seyal* trees (Randall et al., 1989; Qi et al., 1991; Dror et al., 2006). Gum Arabic is an anionic arabinogalactan polysaccharide, comprised of three fractions: 1) a β -(1 \rightarrow 3) galactopyranose (galactan) polysaccharide backbone that is highly branched with β -(1 \rightarrow 6) galactopyranose residues terminating in arabinose and glucuronic acid and/or 4-O-methyl glucuronic acid units (the major fraction, ~89% of the total;

~250 kDa); 2) an arabinogalactan-protein complex, where arabinogalactan chains are covalently linked to a polypeptide backbone (second fraction, ~10% of the total) and 3) the third fraction (~1% of the total) consists of a glycoprotein similar to the arabinogalactan protein complex, except with higher protein levels and comprised of different amino acid sequences (Dror et al., 2006).

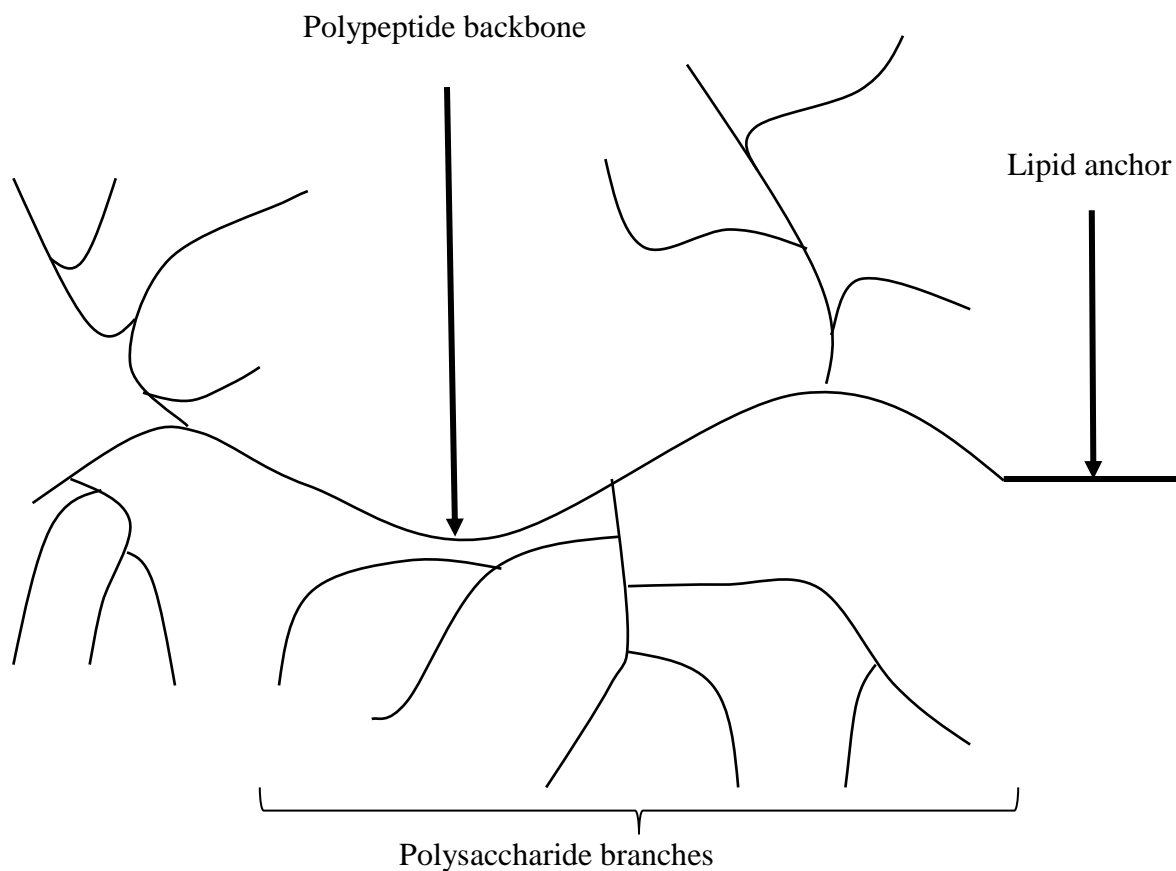


Figure 2.5 Line diagram illustrating the structure of gum Arabic (adapted from Islam et al., 1997)

3. FORMATION OF ELECTROSTATIC COMPLEXES INVOLVING MIXTURES OF LENTIL PROTEIN ISOLATES AND GUM ARABIC POLYSACCHARIDES¹

3.1 Abstract

Formation of electrostatic complexes involving a mixture of lentil protein isolates (LPI) and gum Arabic (GA) as a function of pH (1.50-8.00) and biopolymer mixing ratio (1:4-10:1 LPI:GA) were investigated by turbidimetric analyses during an acid titration. The nature of interactions was also studied in the presence of destabilizing agents (100 mM urea and NaCl), at an elevated temperature (60°C), and as a function of lentil processing (hulled vs. dehulled). Complex formation followed two pH-dependent structure forming events associated with the formation of soluble and insoluble complexes. For the 1:1 LPI:GA ratio, soluble and insoluble complexes formed at pH 5.87 and 3.62, respectively, with maximum formation occurring at pH 3.50. The addition of GA resulted in a shift of LPI's isoelectric point from pH 4.70 to pH 3.17 as chains complexed to the surface of the protein. As the biopolymer mixing ratios increased, critical pHs shifted towards higher pH until reaching the 1:1 mixing ratio, afterwards becoming relatively ratio independent. Complex formation was found to be primarily driven by electrostatic attractive forces with secondary stabilization by hydrogen bonding. Hydrophobic interactions were thought to play a role in the stabilization of LPI-LPI aggregates as part of the formed complexes, rather than in their formation. Removal of the hull resulted in an isolate product with higher surface hydrophobicity than that with the hull. Complex formation shifted slightly to higher pH within the mixture containing isolates derived from dehulled lentil than with the hull on, this was thought to be associated with the higher surface hydrophobicity. Knowledge of the mechanisms driving complex formation within LPI:GA mixtures could lead to improved utilization of this legume as food and/or biomaterial ingredients.

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3.2 Introduction

Interactions between proteins and polysaccharides within foods are of the upmost importance to the food industry because of their influence on ingredient functionality (e.g., emulsification, foaming and gelling/thickening), rheology/texture, structure and processing (Dickinson and Pawlowsky, 1998; Schmitt et al., 1998; Doublier et al., 2000; Weinbreck et al., 2003a,b; Weinbreck et al., 2004a; Mounsey et al., 2008). The overall goal of this research was to further elucidate mechanisms governing associative phase separation (or complex coacervation) involving plant protein-polysaccharide mixtures. Although the exact mechanism is not fully understood, several mixed systems involving plant proteins have been studied, including pea protein-gum Arabic (Ducel et al., 2004; Chourpa et al., 2006; Liu et al., 2009; Liu et al., 2010a,b), pea protein-chitosan (Elmer et al., 2011), pea protein-alginate (Klemmer et al., 2012), canola protein-alginate/ ι -carrageenan (Klassen et al., 2011), and soy protein-pectin (Jaramillo et al., 2011).

In brief, associative phase separation involves the electrostatic attraction of biopolymers with opposing net charges, eventually leading to solution separation into both a solvent-rich and biopolymer-rich phase (Tolstoguzov, 1991; Schmitt et al., 1998; Boral and Bohidar, 2010; Schmitt and Turgeon, 2011; Veis, 2011). Coacervation follows two pH-induced structure forming events associated with the formation of soluble and insoluble complexes during acidification. As proteins take on a net positive charge below its isoelectric point (pI), initial attraction leads to a slight change in turbidity and the formation of soluble complexes (corresponding to a critical pH, denoted as pH_c). As the solution is acidified further, complexes are thought to continuously grow in size and number until reaching a second critical pH (denoted as $\text{pH}_{\phi 1}$) and the formation of insoluble complexes. At this pH, solutions change from a transparent to cloudy sol, and macroscopic changes in turbidity ensues until reaching a maximum (at pH_{opt}), where opposing charged biopolymers reach an electrical equivalence giving a neutral complex (Weinbreck et al., 2004a,b; Liu et al., 2009). As the pH is lowered further, reactive sites on the anionic polysaccharide begin to become protonated leading to the progressive loss of complex structure and turbidity until reaching complete dissolution at $\text{pH}_{\phi 2}$ (Turgeon et al., 2003; Weinbreck et al., 2004a,b; Liu et al., 2009). Although the coacervation process is primarily electrostatically driven, the secondary roles of hydrogen bonding and hydrophobic interactions are less well understood. Furthermore protein-protein aggregation seems to play an important

role in both complex formation and stability (Schmitt et al., 1998; Weinbreck et al., 2003a; Liu et al., 2009).

Charge interactions between the opposing biopolymers must not be too strong; otherwise precipitation of formed complexes may occur rather than coacervation (Boral and Bohidar, 2010). Liquid coacervates are more mobile than precipitates due to the presence of small amounts of entrapped solvent. Coacervates form as entropies associated with biopolymer flexibility and solvent mixing become reduced, to offset the enthalpic contributions associated with the release of water and counterions as complexes form (Schmitt et al., 1999; Singh et al., 2007; Ye, 2008; Klassen et al., 2011). In general, this occurs in mixtures involving a weakly (i.e., those having a relatively low linear charged density or weakly charged reactive site (carboxyl)) charged polyelectrolyte, such as GA and a protein, respectively. In contrast, when electrostatic interactions are strong enough to induce precipitation, counterion and water release are more substantial (Boral and Bohidar, 2010). Furthermore, depending on the strength of interactions between biopolymers, initial soluble complex formation may occur under solvent conditions where both carry similar net charges associated with surface patch binding, or the interactions between a negatively charged polysaccharide with a positive surface patch on a protein's surface (at $\text{pH} > \text{pI}$) (Gupta et al., 2007; Boral and Bohidar, 2010).

The objective of the present study was to investigate the effect of pH and biopolymer mixing ratio on the formation of soluble and insoluble electrostatic complexes within admixtures of lentil protein isolate (LPI) and the anionic polysaccharide, GA. Furthermore, the nature of associative interactions were also explored in the presence of destabilizing agents (e.g., urea and NaCl) at an elevated temperature, and as a function of lentil processing (e.g., hulled vs. dehulled) to better understand mechanisms of interactions.

Lentil represents an economically important and nutritious legume crop (Swanson, 1990; Boye et al., 2010a). Although the functional attributes of LPI have been studied (Lee et al., 2007; Boye et al., 2010a; Roy et al., 2010; Can Karaca et al., 2011), little information is available on their interactions with polysaccharides. Protein levels in lentil are ~25%, with albumins, globulins (legumin and vicilins), glutelins and prolamins representing 16.8%, (44.8% and 4.2%), 11.2% and 3.5% of the total extractable proteins, respectively (Boye et al., 2010a,b). Lentil proteins are also glycosylated (~2.8%) (Boye et al., 2010a,b). During protein isolate production, lentil may be first pre-processed post-harvest to remove the hull (seed coat) prior to being ground

into flour (representing the initial raw material used for extraction). Hulls contain indigestible fibre, polyphenols, tannins and other anti-nutritional factors impact a bitter taste and could impact flour/protein's physicochemical properties (Wang, 2005).

Gum Arabic is a branched carboxylated anionic arabinogalactan polysaccharide (~250 kDa). It is composed of three fractions; the main fraction (~89%) consists of a β -(1→3) galactopyranose (galactan) polysaccharide backbone which is highly branched with β -(1→6) galactopyranose residues terminating in arabinose and glucuronic acid and/or 4-O-methyl glucuronic acid units. The second fraction (~10%) is composed of an arabinogalactan-protein complex, in which arabinogalactan chains are covalently linked to a polypeptide backbone and the last fraction (~1%) comprise of a glycoprotein similar to the arabinogalactan-protein complex, except that it has higher protein levels and different amino acid sequences (Dror et al., 2006).

Improved performance, diversification and utilization of plant proteins is becoming increasingly important in the protein ingredient market as industry searches for an alternative to animal-derived proteins to satisfy consumers perceived fears (e.g., prion disease) and changing dietary preferences/restrictions (Liu et al., 2009). Knowledge of protein-polysaccharide interactions will help facilitate better integration of plant proteins, such as from lentil into this marketplace.

3.3 Material and Methods

3.3.1 Materials

Green lentil seed (CDC Grandora) used to prepare protein isolates (with and without hull) was kindly supplied by the Crop Development Centre, University of Saskatchewan (Saskatoon, SK, Canada), whereas GA (Gum Arabic FT Pre-Hydrated, lot 11229, 2007) polysaccharide was kindly donated by TIC Gums (Belcamp, MD, USA), respectively. Chemical analyses on the GA powder and lentil protein isolates (with and without hulls) were performed (n = 3) according to Association of Official Analytical Chemists (AOAC, 2003) methods; 925.10 (moisture), 923.03 (ash), 920.87 (crude protein), and 920.85 (lipid). Carbohydrate content was determined on the basis of present differential of 100% minus (moisture + lipid + ash + protein). All chemicals used in this study were of reagent grade and purchased from Sigma-Aldrich (Oakville, ON, Canada).

3.3.2 Preparation of protein isolates

Lentil protein isolates were prepared using a method by Papalamprou et al. (2009) with minor modifications. A portion of lentil seeds were processed using a Satak pulse dehuller with a mesh size of 3.0 (SATAK Engineering Co Ltd, Japan) at the Crop Science Grains laboratory, University of Saskatchewan (Saskatoon, SK, Canada) to remove the seed coat. Seeds with and without seed coat, were then ground in a cyclone sample mill with 0.5 mm screen (UDY Corporation, USA) to yield lentil flour. Each flour was subsequently defatted twice using hexane (1: 3 w/v, flour:hexane ratio) for 40-60 min at room temperature (21-23°C). The defatted flour was then dispersed in water (Milli-Q water system, Millipore, Billerica, MA, USA) at a flour: water ratio of 1:10 w/v, adjusted to pH 9.0 using 0.1 M NaOH and allowed to stir for 60 min using a magnetic stirrer. The solution was then centrifuged (1590 x g, 30 min, 4°C) (Beckman J2-HC, Beckman Coulter Canada Inc., Mississauga, ON, Canada) to separate the pellet from the supernatant. The pellet was re-extracted under the same conditions except at flour:water ratio of 1:5 w/v. Both supernatants were pooled, and adjusted to pH 4.6 using 0.1 M HCl. The protein was allowed to precipitate overnight at 4°C. Precipitated proteins were recovered by centrifugation (1590 x g for 30 min at 4°C. The pellet was subsequently washed two more times by dispersing the proteins in water at pH 9.0 for 30 min, followed by reprecipitation at pH 4.6 and centrifugation (1590 x g for 30 min at 4°C). In the final step, the washed pellet was suspended in water adjusted to pH 7.0, then freeze-dried (Labconco Corp., Kansas City, MO, USA) and stored at 4°C.

The LPI (with and without hull) and GA powders were used without further purification. Biopolymer concentrations used in this study was based on the protein and carbohydrate of LPI and GA powders respective content rather than the dry material weight.

3.3.3 Amino acid composition of LPI with and without hull

An analysis of the amino acid content for LPI with and without hull was determined by acid/heat hydrolysis followed by quantification using chromatographic techniques. In brief, 20 mg of LPI were weighed into 20 x 150 mm screw cap Pyrex tubes, 15.00 mL of 6 N HCl was then added followed by flushing of the tubes thoroughly with N₂. The tubes were then capped and placed into an oven at 110°C ± 0.5°C for 20 h. The individual amino acids were then quantified using high performance liquid chromatography via the pico-tag amino acid analysis

system (Waters Corporation, Milford, MA, USA) according to the Association of Official Analytical Chemists (AOAC, 1995) Methods (#985.28, and 988.15) that is based on the procedures of White et al. (1986) and Landry and Delhaye (1993).

3.3.4 Surface hydrophobicity of LPI with and without hull

Surface hydrophobicity of the LPI with and without hull was determined using a modified method of Kato and Nakai (1980) with the fluorescent probe 8-anilino-1-naphthalenesulfonic acid (ANS) (Uruakpa and Arntfield, 2006a; Can Karaca et al., 2011). A stock solutions of LPI (0.01% w/w) was prepared by dispersing the respective powders in 0.01 M sodium phosphate buffer (pH 7.0), and then stirred overnight at 4°C to help facilitate protein solubility. The stock solution was then diluted in the same buffer to obtain protein concentrations of 0.002%, 0.004%, 0.006%, 0.008% and 0.01% (w/v). To 4.0 mL of LPI solutions (0.002-0.01%), 20 μ L of 8 mM ANS solution (in, 0.01 M sodium phosphate buffer pH 7.0) was added. Samples were vortexed (Baxter Diagnostics Inc., Deerfield, IL, USA) for 10 s and then kept in the dark for 15 min. Fluorescence intensities were measured with a FluoroMax-4 Spectrofluorometer (Horiba Jobin Yvon, Kyoto, Japan) at an excitation and emission wavelengths of 390 and 470 nm, respectively, and slit widths of 1 nm. Fluorescence intensity values for the ANS blank and protein blanks (without ANS) were subtracted from the fluorescence intensity of the protein solutions containing ANS. The initial slope of the plot of the net fluorescence intensity versus protein concentration was calculated by linear regression analysis and was used as an index of protein surface hydrophobicity (S_0 -ANS).

3.3.5 Turbidimetric acid pH titrations

Turbidimetric acid pH titrations of homogenous and mixed LPI (with hull only) and GA systems were performed using the modified method of Liu et al. (2009) to identify pHs of critical structure forming events; soluble structure formation (pH_c), insoluble complex formation ($pH_{\phi 1}$), charge neutral complex formation (pH_{opt}) and dissolution of complex ($pH_{\phi 2}$) and, biopolymer and pH conditions where associative and segregative phase separations occurred. All analyses were made at room temperature and at a total biopolymer concentration of 0.05% (w/v). In brief, stock solutions (0.05% w/w; pH 9.0) were initially prepared for LPI (with hull) and GA by dissolving their powders in water under constant stirring (500 rpm) for 2 h at room temperature and then

overnight at 4°C to help facilitate protein solubility. Biopolymer mixtures were then prepared from the stock solution to give the desired total biopolymer concentration (0.05% w/v) and mixing ratio (1:4, 1:2, 1:1, 1.5:1, 2:1, 5:1, and 10:1 LPI (with hull):GA on a weight-by-weight basis). Turbidimetric titration upon acidification was achieved via the addition of an internal acidifier [0.05% (w/w) glucono- δ -lactone, GDL] to slowly lower the mixture pH from 9.0 to 3.9, followed by the drop wise addition of HCl. Diluting effects were kept to a minimum using a gradient of HCl concentrations based on pH (0.05 M > pH 3.3; 0.50 M > pH 2.7; 1.00 M > pH 2.2; 2.00 M > pH 1.5). Dilution was thought to not significantly influence complex formation because critical pH values (pH_c , $pH_{\phi 1}$, and pH_{opt}) measured in this study corresponded to conditions at which no or minimal HCl addition occurred (less than 4% change in volume over the complete titration). Changes in the optical density (O.D.) of the solutions were recorded over a pH range of 8.0-1.5 using a ultraviolet-visible spectrophotometer (Genesys 10, Thermo Scientific, Fair Lawn, NJ, USA) at 600 nm using plastic cuvettes (1 cm path length). Structure-forming transitions (pH_c , $pH_{\phi 1}$, and $pH_{\phi 2}$) were determined graphically as the intersection point of two curve tangents (Weinbreck et al., 2004a; Liu et al., 2009; Klemmer et al., 2012), whereas pH_{opt} corresponded to the maximum optical density at 600 nm. O.D. was measured in triplicate, using separate sample stock solutions and the critical pH values were reported as the mean value \pm standard deviation ($n = 3$). Homogenous solutions of LPI (with hull) and GA were used as blanks under the same solvent conditions and at a biopolymer concentration of 0.05% (w/w).

In a separate study, turbidimetric acid pH titrations were performed on a 1:1 LPI (with hull):GA mixture at a total biopolymer concentration of 0.05% (w/w) in the presence of destabilizing salts (100 mM NaCl and 100 mM urea, at room temperature) and at elevated temperatures (60°C) to test the nature of interactions. Furthermore, a 1:1 mixture of LPI (dehulled):GA were studied in a similar manner to test the effect of lentil hull components on complex formation. Critical pH's were identified in a similar manner as previously described. All measurements were performed in triplicate using separate stock solutions.

3.3.6 Electrophoretic mobility

The electrophoretic mobility (U_E) for a 1:1 LPI (with and without hull):GA mixture and homogenous biopolymer solutions (LPI with and without hull, GA) were investigated as a function of pH 7.0 to 1.5 using a Zetasizer Nano-ZS90 (Malvern Instruments, Westborough,

MA). Solution pH was lowered in 0.5 pH unit increments through the dropwise addition of HCl (0.1 M > pH 5.0; 0.5 M > pH 3.5; 1.0 M > pH 2.5; 2.0 M > pH 1.5) so as to minimize dilution effect. Samples were prepared as described in the previous section above to a final total biopolymer concentration of 0.05% (w/w). Using the Henry equation (Henry, 1931), the electrophoretic mobility (i.e., velocity of a particle within an electric field) was used to give an estimate of the zeta potential (ζ)

$$U_E = \frac{2\varepsilon \times \zeta \times f(\kappa\alpha)}{3\eta} \quad (\text{eq. 3.1}).$$

where η is the dispersion viscosity, ε is the permittivity, and $f(\kappa\alpha)$ is a function related to the ratio of particle radius (α) and the Debye length (κ). Using the Smoluchowski approximation $f(\kappa\alpha)$ equalled 1.5 (Liu et al., 2009). All measurements were made at room temperature (21-23°C) in triplicate.

3.3.7 Statistical Analysis

A one-way analysis of variance (ANOVA) with a Scheffe post-hoc test was used to measure statistical differences within state diagrams for each critical pH value as a function of biopolymer mixing ratio. A student t-test was used to test for statistical differences between critical pH values in mixtures of LPI (with hull): GA in the presence of 100 mM urea and at an elevated temperature (60°C) relative to the control (LPI with hull), and values between LPI:GA mixtures with and without hull. All statistical analyses were performed using Systat software (SPSS Inc., Ver. 10, 2000, Chicago, IL).

3.4 Results and Discussion

3.4.1 Material characterisation

Proximate analysis results for GA and LPI (with and without hull) materials were as follows: a) GA was comprised of 9.56% moisture, 0.86% protein (%N \times 6.25), 0.11% lipid, 84.28% carbohydrate, and 5.19% ash; b) LPI was composed of 9.67% moisture, 78.45% protein (%N \times 6.25), 0.65% lipid, 7.74% carbohydrate, and 3.49% ash; and c) LPI (dehulled) was composed of 10.14% moisture, 80.46% protein (%N \times 6.25), 0.08% lipid, 5.06% carbohydrate, and 4.23% ash. All levels were reported on a wet weight basis. Although there is no universal classification scheme separating a protein isolate from a concentrate, Pearson (1983) using soy developed a criteria requiring a minimum protein content of 85% on a dry weight basis (6.25

nitrogen conversion factor) to be classified as an isolate. In the present study, LPI with and without hull would have an 86.85% (db) and 89.54% (db), respectively using the 6.25 nitrogen conversion factor. Hence in the present study, materials were deemed an isolate product. Protein levels of the LPI (with and without hull) were similar to those reported by Boye et al. (2010a) and Can Karaca et al. (2011) of 79.1% (wb) and 81.90% (wb), respectively.

The amino acid composition (raw and normalized) of LPI with and without hull is given in Table 3.1. Results indicated that the LPI product without hull (85.0%) had slightly higher protein levels (i.e., as indicated by the sum of all amino acids) than the isolate without hull (78.9%). Approximately, 49%, 34% and 17% of the amino acids were hydrophilic, hydrophobic and neutral, respectively for LPI with and without hulls, suggesting that the protein composition was largely unaffected by the dehulling process (Table 3.1).

Surface hydrophobicity (S_o -ANS) values were significantly lower for LPI with hulls (S_o -ANS = 111.39 ± 3.34) than without (S_o -ANS = 257.11 ± 10.41) ($p < 0.001$). Surface hydrophobicity reflects the average amount of exposed hydrophobic groups on the protein's surface as measured by the ANS-fluorescence probe method (Uruakpa and Arntfield, 2006a; Can Karaca et al., 2011). The intensity of the fluorescence intensity reflects the affinity for the ANS probe to the protein (or its ability to bind), a property influenced by the specific amino acids exposed, steric restrictions on the surface contour, and protein conformation. The lower S_o -ANS values in the LPI (with hulls) material may reflect a different level protein aggregation which affects the affinity of the probe to the surface and/or buried hydrophobic sites within the interior of the aggregate structures.

In terms of the surface charge, LPI with and without hulls were found to carry a negative net charge at solution pHs above its isoelectric point (pI; zeta potential = 0 mV), and a positive charge below. The pI was found to be close for both LPI with and without hulls, occurring at pH 4.70 and 4.80, respectively (Figure 3.1). Gum Arabic was found to carry a negative charge over the majority of the pH range until pH 2.07 (Figure 3.1), where below which carboxyl sites along the polysaccharide backbone becomes protonated. Can Karaca et al. (2011) reported zeta potentials between ~-20-23 mV for isoelectric precipitated and salt extracted LPI (with hulls) at pH 7.0, which was comparable to the present study. Liu et al. (2009) reported similar net neutrality for GA at pH 1.88.

Table 3.1 Raw and normalized (to 100% protein) amino acid composition of lentil protein isolates (LPI) produced with and without hulls. Data represent the mean of duplicate samples (n=2).

Amino acids	<u>Raw data</u>		<u>Normalized data</u>	
	LPI with hull (%, w/w)	LPI without hull (%, w/w)	LPI with hull (%, w/w)	LPI without hull (%, w/w)
<i>Hydrophilic</i>				
Aspartic acid + asparagine	9.61	10.50	12.20	12.40
Lysine	5.52	6.05	6.99	7.12
Glutamic acid + Glutamine	13.20	14.10	16.70	16.60
Proline	3.35	3.47	4.24	4.08
Arginine	7.03	7.37	8.91	8.67
(Sub-total)			49.04	48.87
<i>Hydrophobic</i>				
Alanine	3.21	3.13	4.07	3.68
Cysteine	0.58	0.65	0.73	0.76
Tyrosine	3.05	3.19	3.86	3.75
Valine	3.84	4.11	4.86	4.84
Methionine	0.75	0.77	0.95	0.91
Isoleucine	3.58	4.07	4.54	4.79
Leucine	6.53	7.00	8.27	8.24
Phenylalanine	4.57	5.03	5.79	5.92
Tryptophan	0.65	0.69	0.82	0.81
(Sub-total)			33.89	33.70
<i>Neutral</i>				
Threonine	2.76	3.54	3.50	4.16
Histidine	2.35	2.35	2.98	2.76
Glycine	3.12	3.07	3.95	3.61
Serine	5.24	5.92	6.64	6.96
(Sub-total)			17.07	17.49
Total	78.90	85.00	100.00	100.00

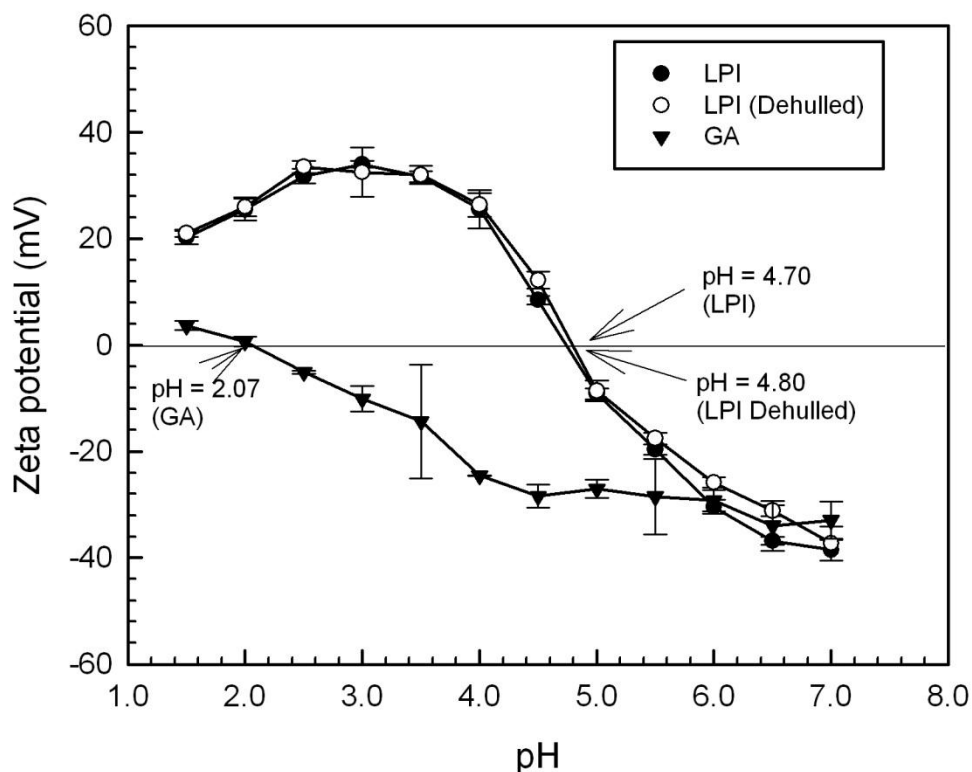


Figure 3.1 Mean zeta potential (mV) of lentil protein isolates (LPI) with and without hulls, and gum Arabic (GA) as a function of pH. Data represent the mean \pm standard deviation ($n = 3$).

3.4.2 Complex coacervation

3.4.2.1 Effect of pH

Changes in O.D. during an acid pH titration were investigated for LPI (with hull) and GA solutions alone, as well as for a mixed LPI (with hull):GA system at a 1:1 biopolymer mixing ratio (Figure 3.2a). For LPI (with hull) alone, a rapid increase in O.D. was observed at pH \sim 5.40, followed by a plateau between pHs \sim 5.00 and 3.40 (corresponding to a maximum O.D. of 0.390), before decreasing in magnitude at pH \sim 2.50. The rise in O.D. is believed to be associated with the formation of LPI-LPI aggregates near its pI (4.70) where net charge reaches neutrality. Whereas, GA showed no change in O.D. over the pH range tested (Figure 3.2a).

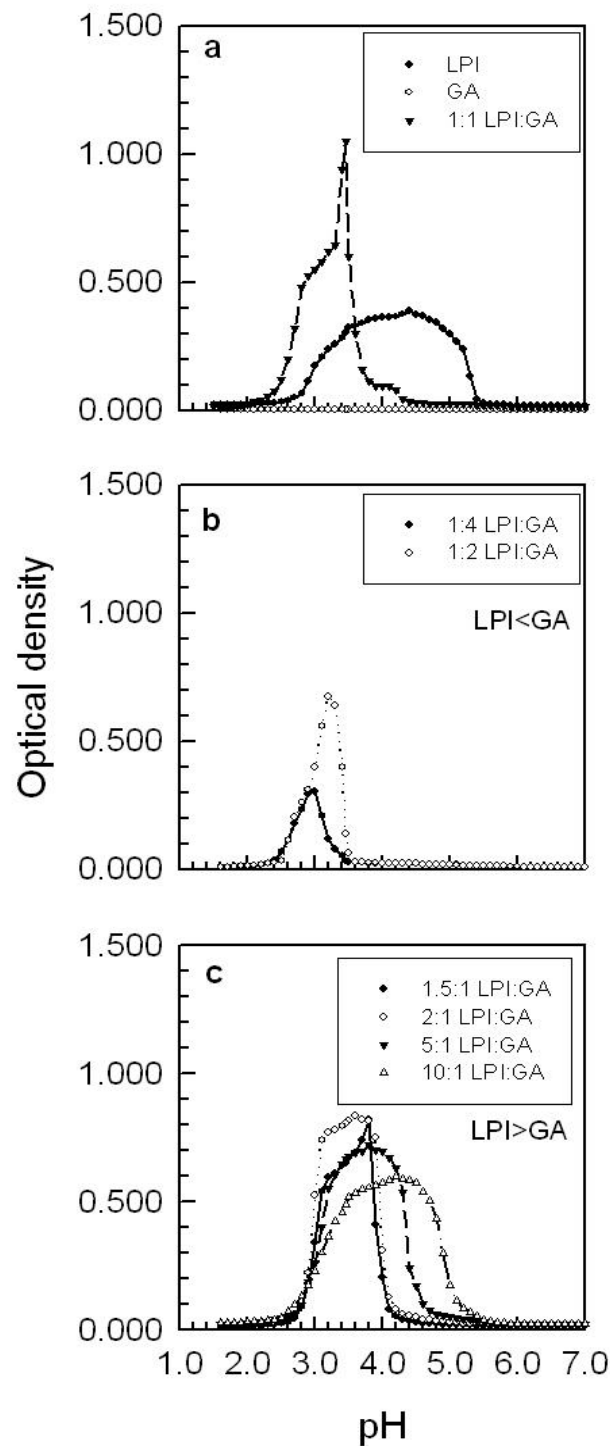


Figure 3.2 Mean turbidity curves for LPI (with hull) and GA alone, and a mixed LPI:GA system (1:1 mixing ratio) as a function of pH (a), and as a function of pH and biopolymer content for systems with LPI:GA mixing ratios <1 (b) and >1 (c) ($n = 3$).

For the mixed LPI (with hull):GA system, both macromolecules carried an overall net negative charge at $\text{pH} > \text{pI}$ leading to electrostatic repulsion between chains. Due to the dilute nature of the dispersion, biopolymers are thought to remain co-soluble rather than segregate into separate liquid-liquid phases. During acidification, the protein's surface begins to take on a positive charge. At $\text{pH} < \text{pI}$, the protein carries an overall net positive charge, and experiences electrostatic attractive forces with the anionic GA chains (Weinbreck et al., 2003a; Liu et al., 2009). Interactions follow two structure-forming events associated with the formation of soluble and insoluble electrostatic complexes.

In the present study, soluble LPI (with hull):GA complexes formed at pH 5.87 (denoted as pH_c), as evident by a slight increase in O.D. with acidification (Figure 3.2a). As pH decreased, a second event associated with the formation of insoluble complexes was evident at pH 3.62 (denoted as $\text{pH}_{\phi 1}$) (Figure 3.2a). Interactions between the macromolecules are thought to arise between the GA chains and positively charged patches on the protein's surface, since at this pH both molecules carry an overall negative charge (i.e., $\text{pH}_c > \text{pI}$). The occurrence of complexation at $\text{pH} > \text{pI}$, has been previously reported for β -lactoglobulin-acacia gum (Schmitt et al., 1999), whey protein-GA (Weinbreck et al., 2003a), pea protein isolate-GA (Liu et al., 2009), soy protein-pectin (Jaramillo et al., 2011), gelatin-agar (Boral and Bohidar, 2010) and gelatin-chitosan (Gupta et al., 2007).

At $\text{pH}_{\phi 1}$ for the LPI (with hull):GA mixture, the O.D. increases sharply in magnitude, and the solution changes from transparent to a cloudy dispersion. The rise in O.D. continues until reaching a maximum of 1.050 at pH_{opt} (3.50) (Figure 3.2a). Near this maximum, macromolecules are thought to reach their electrical equivalent mixing ratio, where charges cancel so as to give an overall neutralization of the complexes (Schmitt et al., 1999; Weinbreck et al., 2003a; Liu et al., 2009). At $\text{pHs} < \text{pH}_{\text{opt}}$, O.D. declines as electrostatic attractive forces begin to break between macromolecules as carboxyl site on the GA backbone progressively becomes protonated. The dissolution of complexes is complete near pH 2.40 as evident by the reduced O.D. in Figure 3.2a.

The presence of GA leads to an initial suppression of protein aggregation, as evident by a minimal O.D. between $\text{pHs} > \sim 4.50$ in the mixed macromolecules profile. It was presumed that complexes form between GA chains and small LPI-LPI aggregates or clusters rather than individual LPI molecules, due to overlapping O.D. curves of LPI (with hull) alone and that of the mixed (Figure 3.2a) system. These aggregates are thought to increase the stability of complexes

from disassociating soon after pH_{opt} , where in the present study a significant shoulder was evident for the LPI (with hull): GA system between pHs of 2.80 and 3.30 (Figure 3.2a). Liu et al. (2010a) investigated the nature of interactions involved with complex formation within admixtures of pea protein isolate and GA. The authors carried a similar turbidimetric analysis as a function of pH and temperature and found that the shoulder increased as reaction temperatures were raised. Furthermore, complex structures became larger at elevated temperatures as measured by confocal laser scanning microscopy (Liu et al., 2010a). The authors postulated that the shoulder was associated with increased complex stabilization due to increased hydrophobic interactions occurring within the pea protein aggregate which electrostatically was bound to GA.

3.4.2.2 Effect of biopolymer mixing ratio

The effect of biopolymer mixing ratio on complex formation within admixtures of LPI (with hull) and GA was also investigated as a function of pH during an acid titration, at ratios where either GA (1:4 and 1:2 LPI:GA) or LPI (1.5:1, 2:1, 5:1 and 10:1 LPI:GA) were dominant. Mixing ratios influence the charge balance between proteins and polysaccharides, the intensity of interactions, and the degree of self-aggregation and therefore play an important role in the complexation of proteins and polysaccharides (Ye, 2008; Liu et al., 2009). When mixing ratios were <1:1, turbidity curves shifted to lower pHs, and the O.D. magnitude at pH_{opt} decreased as the GA level increased, suggesting that charge repulsion was restricting LPI-LPI aggregate and complex formation (Figure 3.2a,b). Furthermore, the shoulder observed in the 1:1 LPI:GA ratio was absent, presumably due to the lower amount of LPI-LPI aggregation. In contrast, at LPI:GA mixing ratios >1:1, turbidity curves shifted to higher pH and the O.D. magnitude at pH_{opt} declined as ratios increased up to 10:1 (maximum ratio examined) (Figure 3.2c). Liu et al. (2009) and Schmitt et al. (1999) both observed similar results for pea protein isolate-GA and β -lactoglobulin-acacia gum mixtures, respectively, where protein concentration was in excess to the available sites on the polysaccharide molecules.

Phase diagrams describing mixing ratio dependence on critical pH values (pH_c , $\text{pH}_{\phi 1}$, pH_{opt} and $\text{pH}_{\phi 2}$) and maximum O.D. are given in Figure 3.3. As mixing ratios increased from 1:4 to 1:1 (LPI:GA), pH_c values shifted to higher pHs ($p < 0.001$) until becoming ratio independent between 1:1 and 10:1 ($p > 0.05$) (Figure 3.3a). In contrast, both $\text{pH}_{\phi 1}$ and pH_{opt} shifted to higher pHs over the complete ratios range, however the rate of change was much greater between ratios

of 1:4 and 1.5:1 LPI:GA, than it was between 1.5:1 and 10:1 (Figure 3.3a). The pH corresponding to the dissolution of complexes (pH_{ϕ_2}) was found to be independent of mixing ratio ($p > 0.05$) (Figure 3.3a). Phase diagrams are important in terms of understanding pH regions where different types of electrostatic complexes (i.e., soluble versus insoluble) are present.

Differing trends have been reported in literature to describe the ratio dependence of pH_c , which could be related to the presence of aggregates, size of the proteins/aggregates and solvent conditions. Similar ratio dependence as found in the present study has been reported for pea protein isolate-GA (Liu et al., 2009), pea protein isolate-alginate (Klemmer et al., 2012), canola protein isolate-alginate/ ι -carrageenan (Klassen et al., 2011) and gelatin (Type-A and Type-B)-agar (Singh et al., 2007) systems; attributed to the interaction between protein-protein aggregates with polysaccharide chains. In contrast, research on milk protein-polysaccharides with aggregates removed by filtration revealed pH_c to be independent of mixing ratio. This result was also reported by Weinbreck et al. (2003a,b) for whey protein (aggregate-free)-polysaccharide, and Girard et al. (2002) for lactoglobulin (aggregate-free)-pectin systems. The authors in each case presumed that soluble complexes formed between individual proteins and polysaccharides. Mixing ratio dependent trends associated with pH_{ϕ_1} are quite prevalent in literature, where values shift to higher pHs to some ratio (system dependent), and then either plateaus or continues to shift to higher pHs only at a slower rate. Similar trends as the present study were present in all the aforementioned researches.

Figure 3.3b illustrates the ratio dependence of the maximum O.D. at pH_{opt} , where values increased from a 1:4 to 1:1 LPI:GA ratio ($p < 0.001$), then declined as ratios increased to 10:1 LPI:GA. The O.D. maximum has been identified by others as the ratio where maximum macromolecular complexation occurs (Girard et al., 2002; Weinbreck et al., 2003a; Liu et al., 2009) and corresponds to the stoichiometric equivalent of proteins/polysaccharides at that ratio (Girard et al., 2002; Weinbreck et al., 2003a; Liu et al., 2009). At the 1:1 LPI:GA ratio, charges on the lentil proteins are thought to be saturated with GA chains. At mixing ratios $> 1:1$ LPI:GA, proteins are in excess giving rise to an increased amount of LPI-LPI aggregation and less complex formation. Similar effects were reported for whey protein isolate-(ι -, κ - and λ -type) carrageenan (Stone and Nickerson, 2012), pea protein isolate-GA (Liu et al., 2009), pea protein isolate-alginate (Klemmer et al., 2012) and canola protein isolate-alginate/ ι -carrageenan (Klassen et al., 2011).

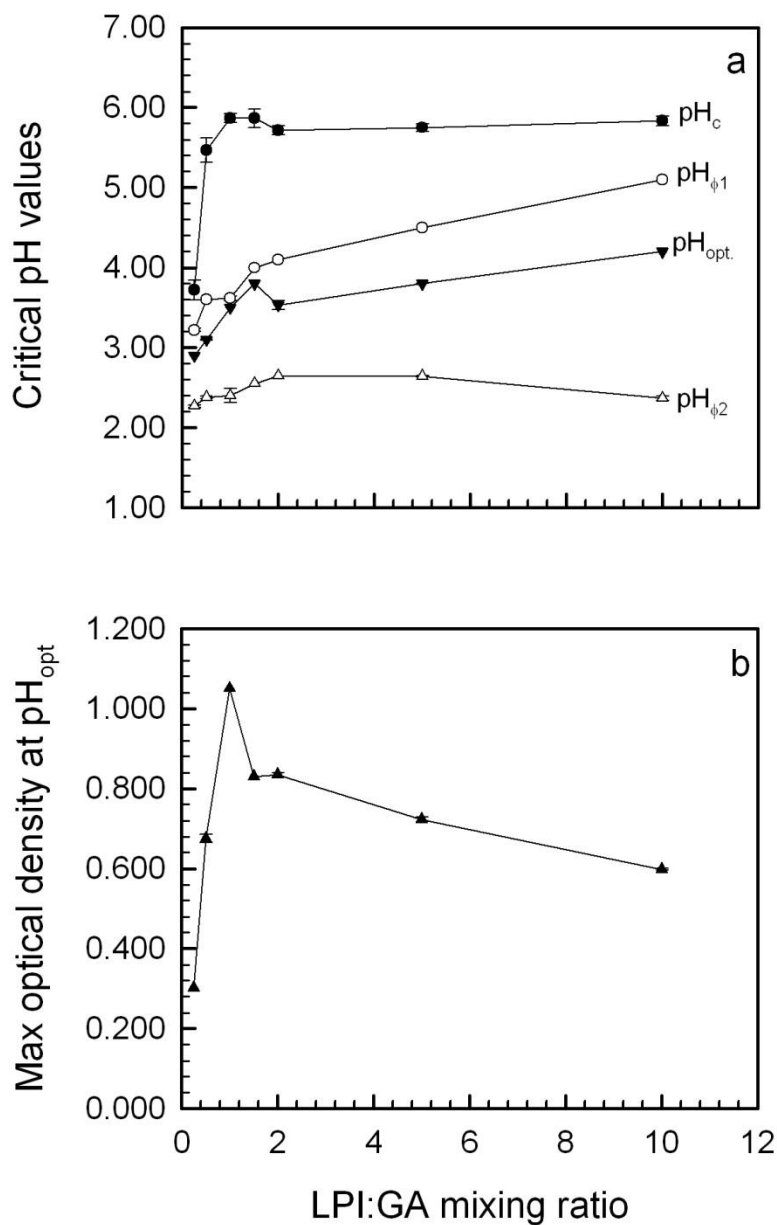


Figure 3.3 Critical pH values associated with structure forming events during coacervation (pH_c , $pH_{\phi 1}$, pH_{opt} and $pH_{\phi 2}$) (a) and maximum optical density (b) in LPI:GA mixtures as a function of biopolymer mixing ratio. Data represent the mean \pm standard deviation ($n = 3$).

Electrophoretic mobility of a 1:1 LPI:GA mixture as a function of pH indicated that net neutrality (zeta potential = 0 mV) occurred at pH 3.17; above and below this value the complex carried either a net negative or positive charge, respectively (Figure 3.4). Net neutrality in the present system occurred near the pH_{opt} value of 3.50 (Figure 3.2a). The shift in pH corresponding to net neutrality from 4.70 (LPI with hulls, alone) to 3.17 reflects the charge contribution from GA. These results suggest that the reactive site on the GA molecule (carboxyl group) has a direct impact on the surface characteristics of the formed complex. A similar shift in net neutrality with complex formation was reported by Stone and Nickerson (2012) in whey protein isolate-(ι -, κ - and λ -type) carrageenan and in various plant protein–polysaccharide mixtures (Liu et al., 2009; Klassen et al., 2011; Klemmer et al., 2012).

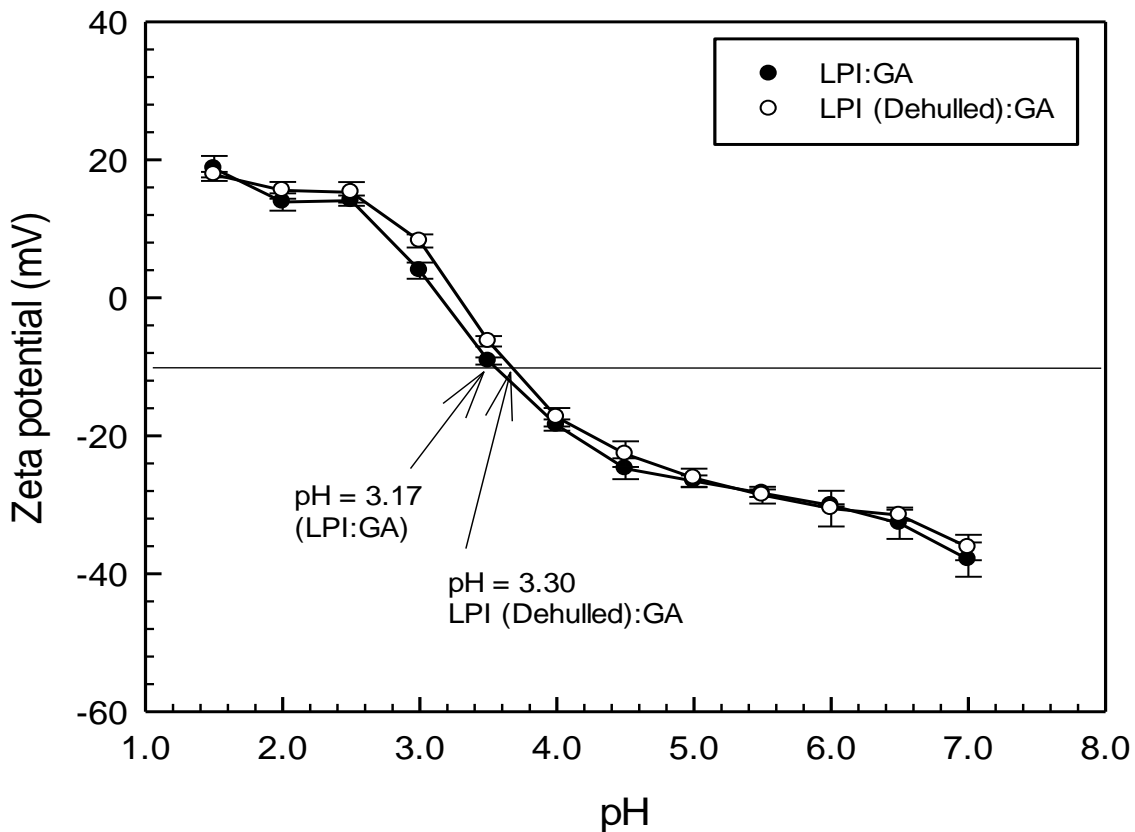


Figure 3.4 Mean zeta potential (mV) for a 1:1 (LPI with and without hulls):GA mixture as a function of pH. Data represent the mean \pm standard deviation ($n = 3$).

3.4.3 Nature of LPI (with hull):GA interactions

Complex formation was also investigated for a 1:1 LPI (with hull):GA ratio during a pH acid titration in the presence of destabilizing agents (100 mM NaCl and 100 mM urea, at room temperature) and at an elevated temperature (60°C) so as to discern the nature of secondary protein-polysaccharide interactions. Overall, complex formation is generally conceived to be driven by electrostatic attractive forces between two opposing charged polymers (Tolstoguzov, 1991; Schmitt et al., 1998; Weinbreck et al., 2003a; Liu et al., 2009; Schmitt and Turgeon, 2011; Veis, 2011), however the role of secondary forces such as hydrogen bonding and hydrophobic interactions are less understood in terms of both formation and stability. Due to difficulties delineating the contributions from each intermolecular force/interaction, solvent manipulation is commonly used as a means to have one dominate over another (Uruakpa and Arntfield, 2005; Kaibara et al., 2000; Weinbreck et al., 2004a). Urea acts to disrupt hydrogen bonding and hydrophobic interactions (Uruakpa and Arntfield, 2006b; Liu et al., 2010a), whereas elevated temperatures acts to both disrupt hydrogen bonds and enhance hydrophobic interactions (Liu et al., 2010a). In contrast, the presence of salts influences electrostatic forces within the system through a charge screening effect of the electric double layers of the two biopolymers. Changes to the turbidimetric profiles (and critical pH values) would aid in determining the significance of each force/interaction in complex formation.

In the presence of 100 mM NaCl, complex formation between LPI (with hulls) and GA was inhibited, as the dissociated Na^+ and Cl^- ions in solution acted to screen charges on GA and LPI's surface to disrupt electrostatic attractive forces between the two biopolymers (Figure 3.5) (Schmitt et al., 1999; Uruakpa and Arntfield, 2006b; Liu et al., 2009; Liu et al., 2010a). The addition of NaCl caused the turbidity curve to decrease substantially relative to the control (1:1 LPI:GA ratio at 21-23°C without destabilizing agents) and broaden the range between pH ~2.00 and 6.00 (Figure 3.5) to become more comparable to LPI alone (Figure 3.2a).

The addition of 100 mM urea caused an overall shift in the turbidity curve towards more acidic pH relative to the control and a drop in O.D. magnitude at pH_{opt} indicating a loss of complex structure stability due to the breakage of hydrogen bonds. However, since the turbidity profile followed a similar pattern as the control, hydrogen bonding was considered to act as a secondary force influencing complexation. With the exception of pH_c (pH 5.90), all critical pH values ($\text{pH}_{\phi 1}$, pH_{opt} and $\text{pH}_{\phi 2}$) significantly shifted to lower pH relative to the control ($p < 0.05$)

(Table 3.2). These values corresponded to pHs of 3.58, 3.40 and 2.32, respectively (Table 3.2). Maximum O.D. at pH_{opt} also was found to decrease significantly from 1.050 (control) to 0.842 (Table 3.2) ($p<0.05$).

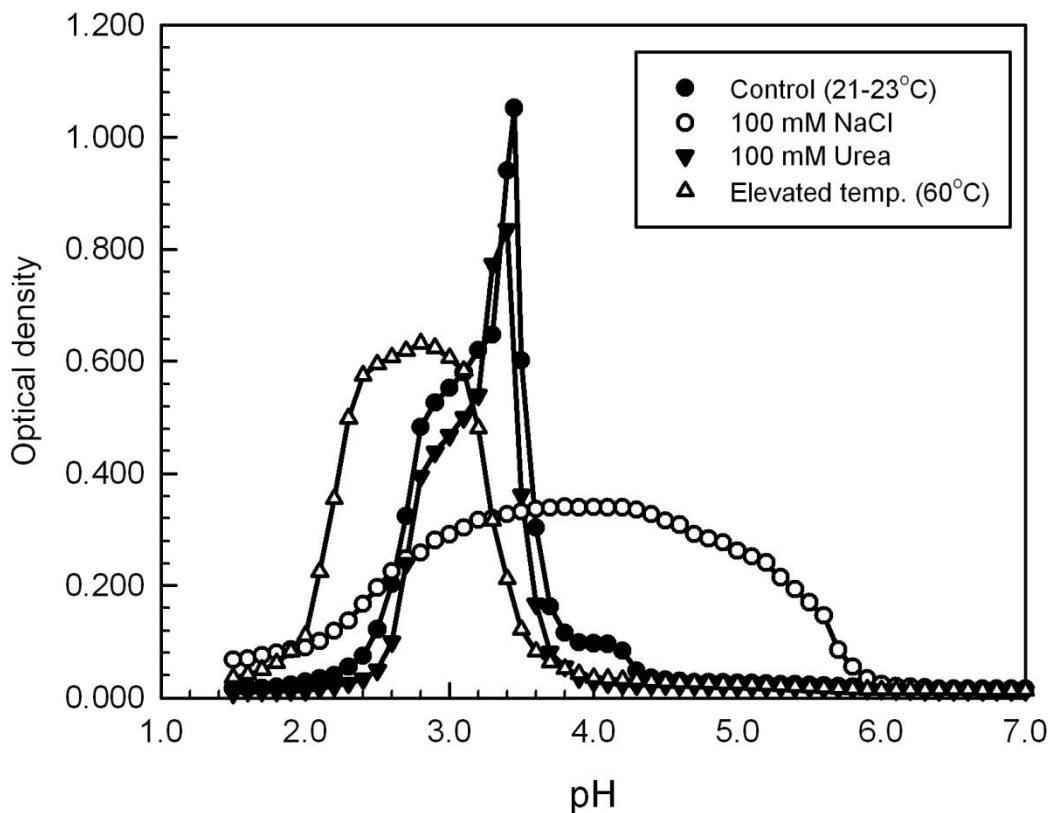


Figure 3.5 Mean turbidity curves of a 1:1 LPI:GA mixture as a function pH, with (100 mM NaCl; 100 mM urea) and without (control, 21-23°C) destabilizing agents, and at an elevated temperature (60°C) ($n=3$).

In the present study, turbidity curves shifted towards more acidic pH at 60°C relative to the control (21-23°C), along with a decrease in maximum O.D. at pH_{opt} from 1.050 to 0.633 (Figure 3.5, Table 3.2). The turbidity profile was also more Gaussian or bell-shaped in nature versus the control which skewed left towards acidic pHs and contained a prominent shoulder between pH 2.80 and 3.30 (Figure 3.5). The broaden nature of the 60°C curve is thought to be associated with enhanced stabilizing hydrophobic interactions within the LPI-LPI aggregated. Critical pH values associated with $pH_{\phi 1}$, pH_{opt} and $pH_{\phi 2}$ were found to be 3.50, 2.80 and 1.97, respectively, which were significantly less than the control ($p<0.05$) (Table 3.2). The formation

of soluble complexes at pH_c was similar to the control and unaffected by temperature ($p>0.05$) (Table 3.2).

Table 3.2 Optical density at critical pH values (pH_c , $pH_{\phi 1}$, pH_{opt} and $pH_{\phi 2}$) and maximum O.D. values for (a) LPI (with hull):GA mixtures (control, 21-23°C, 1:1 mixing ratio) with and without destabilizing agents (100 mM NaCl; 100 mM Urea), and at an elevated temperature (60°C); and (b) LPI (with and without hull):GA mixtures (1:1 mixing ratio). Data represent the mean \pm standard deviation ($n = 3$).

Conditions	pH_c	$pH_{\phi 1}$	pH_{opt}	$pH_{\phi 2}$	O.D. _{max}
<i>(a) Nature of interactions</i>					
21-23°C	5.87 ± 0.06	3.62 ± 0.00	3.50 ± 0.00	2.40 ± 0.00	1.050 ± 0.000
100 mM NaCl	-	-	-	-	-
100 mM Urea	5.90 ± 0.00	3.58 ± 0.03	3.40 ± 0.00	2.32 ± 0.03	0.842 ± 0.010
60°C	5.90 ± 0.00	3.50 ± 0.00	2.80 ± 0.00	1.97 ± 0.03	0.633 ± 0.011
<i>(b) LPI:GA mixtures</i>					
With hull	5.87 ± 0.06	3.62 ± 0.00	3.50 ± 0.00	2.40 ± 0.00	1.050 ± 0.000
Without hull	5.73 ± 0.06	3.85 ± 0.00	3.65 ± 0.00	2.75 ± 0.00	1.020 ± 0.017

(-) Not observed

The observed change in the turbidity profile curve for LPI:GA at 60°C is presumed to reflect the disruption of hydrogen bonding which influences both the formation and stability of complexes. However at this elevated temperature hydrophobic interactions seem to play a greater role in LPI-LPI aggregate stability as indicated a broaden shoulder within the turbidity profile at $pH_s < pH_{opt}$ relative to the control (Figure 3.5). Similar nature of interactions in response to destabilizing agents and elevated temperatures were reported for pea protein isolate-GA (Liu et al., 2010a) and canola protein isolate-alginate/ ι -carrageenan (Klassen et al., 2011). Girard et al. (2002) reported that at elevated temperatures, the yield of β -lactoglobulin and, low- and high-methylated pectin complexes declined, suggesting that hydrogen bonding was involved with complex formation. Secondary roles of hydrogen bonding in the complexation of proteins and polysaccharides have been previously reported for admixtures of xanthan gum and gelatin (Lii et

al., 2002), alfalfa rubisco and pectin (Antonov and Sochinsky, 2000) and, β -lactoglobulin and pectin (Girard et al., 2002).

3.4.4 Influence of dehulling on LPI:GA coacervation

The effect of dehulling lentil prior to isolate preparation was examined for its effect on the formation of electrostatic complexes with GA at a 1:1 LPI:GA mixing ratio during an acid titration. Turbidity profiles as a function of pH were found to be similar in shape irrespective of the presence of a hull, however mixtures involving LPI (without hulls) shifted to higher pHs relative to those with hulls (Figure 3.6). With the exception of pH_c ($\text{pH } 5.73 \pm 0.06$), all critical pHs values ($\text{pH}_{\phi 1}$, pH_{opt} and $\text{pH}_{\phi 2}$) shifted to higher pH relative to the control (LPI with hull) ($p < 0.05$) (Table 3.2). These values corresponded to pHs of 3.85 ± 0.00 , 3.65 ± 0.00 and 2.75 ± 0.00 , respectively (Table 3.2). Maximum O.D. at pH_{opt} also was found to be similar irrespective of the presence of the hull ($p > 0.05$). Electrophoretic mobility measurements indicated similar isoelectric points (zeta potential = 0 mV) for LPI alone with and without hulls (Figure 3.1). However net neutrality shifted to lower pH for both mixtures involving GA and LPI with (3.17) and without (3.30) hulls (Figure 3.4).

Boye et al. (2010a) reported that processing techniques and extraction conditions influence protein yield, quality, composition and purity. Can Karaca et al. (2011) investigated the physicochemical properties of pH-precipitated and salt-extracted legume protein isolates (e.g., chickpea, lentil, soy and pea) to find the method of processing can drastically effect protein behaviour as ingredients and influence the surface hydrophobicity of the proteins extracted. Higher surface hydrophobicity in proteins is associated with a greater number of hydrophobic patches on the surface (Uruakpa and Arntfield, 2006a; Can Karaca et al., 2011). In the present study, S_o -ANS was reported to be much lower for the LPI with hull ($S_o\text{-ANS} = 111.39$) than LPI without hull ($S_o\text{-ANS} = 257.11$), despite having a similar amino acid composition (Table 3.1) and surface charge (Figure 3.1). In the present study, the shift towards higher pH is presumed to be attributed to either the presence of hydrophobic interactions which might help stabilize complexes during formation or because of the removal of polyphenols, tannins, fibre or other nutritional factors from the flour during dehulling.

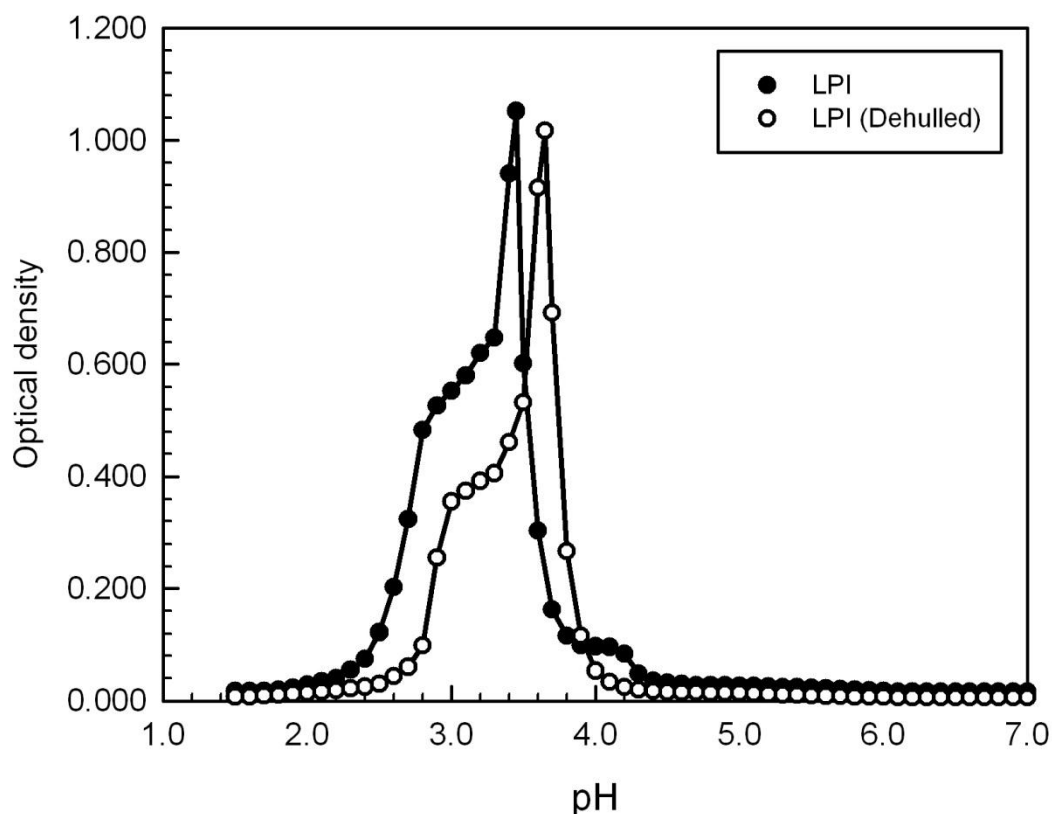


Figure 3.6 Mean turbidity curves for mixed LPI:GA systems (1:1 mixing ratio) as a function of pH using LPI with and without hulls (n = 3).

3.5 Conclusions

Complex coacervation involving LPI and GA followed two structure forming events associated with the formation of soluble and insoluble complexes. Initial interactions occurred slightly above the pI of the LPI where both biopolymers carried similar net negative charges. Macromolecular interactions were proposed to be facilitated through negatively charged GA chains with positively charged patches on the protein's surface. However, the majority of structure formation occurred below the pI of the protein where both biopolymers carried opposing net charges. Complexes are thought to form between GA chains with small LPI-LPI aggregates rather than individual proteins. Complex formation was also found to be primarily due to electrostatic attractive forces with secondary stabilization by hydrogen bonding.

Hydrophobic interactions seemed to play a role in the stability of formed complexes but not in its formation. Removal of the hull prior to isolate production resulted in a protein ingredient with a slightly higher surface hydrophobicity than isolates prepared with the hull. Dehulling of the lentil resulted in complex formation occurring at slightly higher pH than those with the hull. However, it was unclear whether this was due to the higher surface hydrophobicity or the removal of fibre, polyphenols/tannins or other antinutritional factors from the flour.

3.6 Linkage

Complex coacervation between LPI and the weakly charged polysaccharide GA led to structure formation over a narrow pH range. Complexes were formed primarily by electrostatic attraction, with secondary stabilization by hydrogen bonding. Coacervate structures formed at solvent pHs < pI of the protein, where biopolymers were of opposing net charge. The focus of the second study of this research project was to investigate the mechanisms of complex formation existed when LPI was mixed with more highly charged polysaccharides such as GG and κ -CG. The presence and concentration of monovalent (Na^+ , K^+) and divalent (Ca^{2+}) salts were also explored in mixed systems of LPI:GG and LPI: κ -CG in order to study their effects on complex coacervation.

4. FORMATION OF ELECTROSTATIC COMPLEXES WITHIN LENTIL PROTEIN ISOLATE – POLYSACCHARIDE (κ -CARRAGEENAN AND DE-ACYL GELLAN GUM) MIXTURES

4.1 Abstract

Complex formation within lentil protein isolate (LPI)-polysaccharide (κ -carrageenan (κ -CG) and de-acyl gellan gum (GG)) mixtures were investigated as a function of pH (1.50-8.00) and mixing ratio (1:1-30:1; LPI:polysaccharide) by turbidimetric analysis and electrophoretic mobility during an acid titration. Effects of monovalent and divalent salts (NaCl, KCl and CaCl₂) on complex stability were also studied as a function of ionic strength (0-1500 mM). Coacervation typically follows two pH-dependent structure forming events associated with the formation of soluble and insoluble complexes. The addition of anionic polysaccharides to a LPI system (at all concentrations and ratios) resulted in a significant drop in turbidity over the entire pH-range, where LPI aggregation was thought to be significantly inhibited by electrostatic repulsive forces between neighbouring polysaccharide chains. As the biopolymer mixing ratio increased, structure formation was less inhibited. Solution pH corresponding to the formation of soluble and insoluble complexes was found to shift to higher pHs, and turbidity increased as the mixing ratios were raised. The presence of either polysaccharide resulted in a shift in net neutrality (zeta potential = 0 mV) of the formed complexes from pH 4.70 (LPI alone) to pH 4.36, as measured by electrophoretic mobility of a 15:1 LPI: polysaccharide mixture. The addition of salts resulted in the disruption of formed LPI: polysaccharide complexes, as evident by a significant drop in turbidity as ionic strength increased. This effect was more pronounced in the presence of calcium than monovalent ions, and no polysaccharide-ion effects were evident (i.e., greater sensitivity of GG to Ca²⁺ and κ -CG to K⁺).

4.2 Introduction

A greater understanding of protein-polysaccharide interactions in food systems is especially important as it relates to controlling food structure, texture and stability. Depending on the solvent conditions, biopolymer mixtures may undergo segregative or associative phase

separation. The former relates to conditions where biopolymers carry similar net charges and leads to phase separation into both a protein-rich and polysaccharide rich phase. In the case of the latter, biopolymers tend to have opposing net charges, leading to phase separation into both a biopolymer (protein + polysaccharide)-rich and solvent-rich phases (Doublier et al., 2000; Weinbreck et al., 2003a,b; de Kruif et al., 2004).

In mixtures involving proteins with highly charged anionic polysaccharides (e.g., carrageenan, gellan gum and alginate), two pH dependent structure forming events occur with the formation of electrostatic complexes as measured by changes in turbidity during an acid pH titration. Initially, soluble complexes form at a critical pH (denoted as pH_c) near the protein's isoelectric point (pI) as evident by a slight rise in turbidity (Liu et al., 2009). Due to the highly charged nature of the polysaccharide, initial interactions may occur at pH values slightly above pI where biopolymers have a similar net charge due to polysaccharide interactions with positively charged patches on the protein's surface (Klassen et al., 2011). As the pH is lowered, macroscopic phase separation ensues at a critical pH (denoted as $pH_{\phi 1}$) associated with the formation of insoluble complexes. At this point, a sol transitions from transparent to cloudy and a large rise in turbidity occurs (Weinbreck et al., 2004b). Formed electrostatic complexes tend to precipitate and fall out of solution soon after $pH_{\phi 1}$.

The present study aims to better understand mechanisms associated with the formation of electrostatic complexes within mixtures of a lentil protein isolate (LPI) and the anionic polysaccharides; κ -carrageenan (κ -CG) and deacyl gellan gum (GG). Specifically, the study looks at the effect of pH, biopolymer mixing and polysaccharide-type (sulphated vs. carboxylated) on the formation of electrostatic complexes, and whether in the presence of salts, any polysaccharide-ion specificity effects arise. Lentil (*Lens culinaris*) proteins are dominated by salt-soluble globulins comprised of both legumin (hexamer; 11S – Svedberg Unit; ~350-400 kDa) and vicilin (trimer; 7S; ~150-180 kDa) (Boye et al., 2010a,b). κ -Carrageenan is a linear anionic sulphated polysaccharide consisting of repeating disaccharide units of alternating (1→3)- α -D-galactopyranosyl-4-sulphate and (1→4)- β -3,6-anhydro-D-galactopyranosyl monomers (Ould et al., 2000; Nickerson and Paulson, 2004; Nickerson et al., 2004). In contrast, GG is a linear carboxylated polysaccharide consists of repeating tetrasaccharide units of [→3)- β -D-glucopyranosyl- (1→4)- β -D-glucuronopyranosyl- (1→4)- β -D-glucopyranosyl- (1→4)- α -L-rhamnopyranosyl- (1→)]_n. κ -CG and GG are considered to be sensitive to K^+ and Ca^{2+} ions,

respectively (Nickerson and Paulson, 2004). Polysaccharide-ion sensitivities relate to ion-mediated junction zone formation during gelation and increased gel strength.

4.3 Material and Methods

4.3.1 Materials

Green lentil seed (CDC Grandora) used to prepare protein isolates was kindly supplied by the Crop Development Centre, University of Saskatchewan (Saskatoon, SK), whereas GG (Lot # 8K4681A) was kindly donated by CP Kelco (Atlanta, GA, USA). κ -CG (Lot # 0001432063), along with all other chemicals used in this study were purchased from Sigma-Aldrich Ltd. (Oakville, ON, Canada). Proximate composition of the prepared LPI (See Sec. 2.2), GG and κ -CG powders were determined according to AOAC (2003) methods; 925.10 (moisture), 923.03 (ash), 920.87 (crude protein), and 920.85 (lipid). Carbohydrate content was determined on the basis of present differential from 100%. GG was composed of 79.75% carbohydrate, 7.71% moisture, ash [mineral content (w/w): 0.49% sodium, 4.56% potassium, 0.28% calcium, 0.09% magnesium], 0.06% lipid and 0% protein. In contrast, κ -CG was comprised of 70.55% carbohydrate, 8.09% moisture, 21.35% ash [mineral content (w/w): 0.64% sodium, 9.00% potassium, 0.09% calcium, 0.13% magnesium], 0.01% lipid and 0% protein. Biopolymer concentrations used in this study were corrected for protein or carbohydrate content. All materials were used without further purification.

4.3.2 Preparation of protein isolates

Lentil protein isolates were prepared using a method by Papalamprou et al. (2009). In brief, lentil seeds were ground into flour using a cyclone mill equipped with a 0.5 mm screen (UDY Corporation, Fort Collins, CO, USA). The flour was then defatted twice using hexane (1:3 w/v, flour: hexane) for 60 min at room temperature (21-23°C). The defatted flour was dispersed in water (Milli-Q water system, Millipore, Billerica, MA, USA) at a flour:water ratio of 1:10 w/v, adjusted to 9.00 using 0.1 M NaOH and allowed to stir for 60 min using a magnetic stirrer. The dispersion was then centrifuged using at 1590 x g for 30 min at 4°C (Beckman J2-HC, Beckman Coulter Canada Inc., Mississauga, ON, Canada) to collect the supernatant. The pellet was re-extracted under the same conditions except at a flour:water ratio of 1:5 w/v. Both supernatants were pooled, and adjusted to pH 4.6 using 0.1 M HCl. The protein was allowed to

precipitate overnight at 4°C. Precipitated proteins were recovered by centrifugation 1590 x g for 30 min at 4°C. The pellet was subsequently washed twice by dispersing the proteins in water at pH 9.00 for 30 min, followed by precipitation at pH 4.60 and centrifugation (1590 x g for 30 min at 4°C). In the final step, the washed pellet was suspended in water at pH 7.00, and then freeze-dried (Labconco Corp., Kansas City, MO, USA) and stored at 4°C. The LPI was comprised of 78.45% protein (%N \times 6.25), 9.67% moisture, 0.65% lipid, 7.74% carbohydrate, and 3.49% ash.

4.3.3 Turbidimetric acid pH titrations

Formation of soluble and insoluble complexes were investigated by turbidimetric acid pH titrations of individual and mixed LPI and GG/ κ -CG systems as described by Liu et al. (2009). All analyses were made at room temperature and at a total biopolymer concentration of 0.05% (w/v). Stock solutions (0.05%, w/w; pH 9.00) were initially prepared for LPI, GG and κ -CG by dissolving their powders in water under constant stirring (500 rpm) for 2 h at room temperature and then overnight at 4°C. Biopolymer mixtures were then prepared using the stock solution to give the desired total biopolymer concentration (0.05% w/v) and mixing ratio (1:1, 2:1, 5:1, 10:1, 15:1, 20:1 and 30:1 LPI: GG/ κ -CG, on a weight-by-weight basis. Turbidimetric titration upon acidification was achieved via the addition of an internal acidifier [0.05% (w/w) glucono- δ -lactone, GDL] to slowly lower the mixture pH from 9.0 to 3.9, followed by the drop wise addition of HCl. Diluting effects were kept to a minimum using a gradient of HCl concentrations based on pH (0.05 M > pH 3.3; 0.50 M > pH 2.7; 1.00 M > pH 2.2; 2.00 M > pH 1.5). Dilution was thought to not significantly influence complex formation because critical pH values (pH_c , $pH_{\phi 1}$) measured in this study corresponded to conditions at which no or minimal HCl addition occurred (less than 4% volume change over the complete titration). Changes in the optical density (O.D.) were recorded over a pH range of 8.00-1.50 using an ultraviolet-visible spectrophotometer (Genesys 10, Thermo Scientific, Fair Lawn, NJ, USA) at 600 nm using plastic cuvettes (1 cm path length). Structure-forming transitions (pH_c , $pH_{\phi 1}$) were determined graphically as the intersection point of two curve tangents (Weinbreck et al., 2004a; Liu et al., 2009; Klemmer et al., 2012). All turbidity scans were done in triplicate, using separate stock solutions and the critical pH values were reported as the mean value \pm one standard deviation ($n = 3$). Individual solutions of LPI, GG and κ -CG were used as controls under the same solvent conditions and at a biopolymer concentration of 0.05% (w/v).

In a separate study, the effect of NaCl, KCl and CaCl₂ concentration (0 - 1.5 M) on turbidity was measured for an individual LPI solution and a 15:1 LPI: polysaccharide mixture at a total biopolymer concentration of 0.05% (w/v) and pH of 4.35. This pH corresponded to where mixtures became electrically equivalent (See Results and Discussion Sec.). All measurements were performed in triplicate.

4.3.4 Electrophoretic mobility

Zeta potential (mV) for individual and mixed (15:1 ratio) LPI-polysaccharide solutions were investigated by measuring the electrophoretic mobility (U_E) as a function of pH 7.00 to 1.50 using a Zetasizer Nano-ZS90 (Malvern Instruments, Westborough, MA, USA). Solution pH was lowered in 0.5 pH unit increments through the drop wise addition of HCl (0.1 M > pH 5.0; 0.5 M > pH 3.5; 1.0 M > pH 2.5; 2.0 M > pH 1.5). All solutions were prepared as previously described. Using the Henry equation (Henry, 1931), the electrophoretic mobility (i.e., velocity of a particle within an electric field) was used to give an estimate of the zeta potential (ζ)

$$U_E = \frac{2\varepsilon \times \zeta \times f(\kappa\alpha)}{3\eta} \quad (\text{eq. 3.1})$$

where η is the dispersion viscosity, ε is the permittivity, and $f(\kappa\alpha)$ is a function related to the ratio of particle radius (α) and the Debye length (κ). Using the Smoluchowski approximation $f(\kappa\alpha)$ equalled 1.5 (Liu et al., 2009). All measurements were made at room temperature (21-23°C) in triplicate.

4.3.5 Statistical Analysis

A one-way analysis of variance (ANOVA) with a Scheffe post-hoc test was used to measure statistical differences within state diagrams for each critical pH value as a function of biopolymer mixing ratio. All statistical analyses were performed using Systat software (SPSS Inc., Ver. 10, 2000, Chicago, IL).

4.4 Results and Discussion

4.4.1 Individual biopolymer solutions

Changes to O.D. as a function of pH for individual LPI, κ -CG and GG solutions are given in Figure 4.1A. LPI experienced a rapid rise in O.D. near pH ~5.50 up to a maximum O.D. of 0.390 at pH 4.40, before decreasing to baseline near pH 2.80. The observed rapid rise in O.D.

is thought to be associated with formation of LPI-LPI aggregates as a result of reduced electrostatic repulsive forces between individual proteins near its pI. Electrophoretic mobility measurements indicated that LPI reached net neutrality at pH 4.70 (Figure 4.1B), and at pH>pI and pH<pI LPI carried a net positive and negative charge, respectively. The O.D. for both κ -CG and GG was negligible over the complete pH range (Figure 4.1A). Zeta potentials for both polysaccharides were negative over the pH range tested, with κ -CG exhibiting greater surface charge than GG due to the presence of sulphate groups. Zeta potential values ranged between -5 and -35 mV for GG and, -35 and -60 mV for κ -CG respectively, over the pH range of 1.50 and 7.00 (Figure 4.1B).

4.4.2 Formation of electrostatic LPI: κ -CG and LPI:GG complexes

The formation of electrostatic complexes within mixtures of LPI: κ -CG and LPI:GG were investigated by turbidimetric analysis as a function of biopolymer mixing ratio (1:1 to 30:1 LPI: polysaccharide) and pH (1.50-8.00). In the case of the LPI: κ -CG mixture at the 1:1 mixing ratio, O.D. was significantly lower over the complete pH range, and its maximum was shifted to a much lower pH (Figure 4.2A) relative to LPI alone (Figure 4.1A). The presence of the highly charged κ -CG chains were thought to induce significant electrostatic repulsive forces between neighbouring polysaccharide chains to restrict both the size and number of LPI-LPI aggregates from being formed. It was presumed that even LPI: κ -CG complexes (at the 1:1 ratio) remained highly negatively charged over the majority of the pH range (although not measured). As the biopolymer mixing ratio increased (i.e., greater proportion of LPI present), turbidity profiles shifted back towards higher pH and O.D. increased in magnitude until reaching comparable levels at the 30:1 mixing ratio (Figure 4.2A) as to the individual LPI solution (Figure 4.1A). The suppression of LPI-LPI aggregation observed as a function of biopolymer mixing ratio, reflects the reduced electrostatic repulsive forces between polysaccharide chains as their concentration relative to LPI is reduced. However, at all mixing ratios, precipitation of the electrostatic complexes occurred.

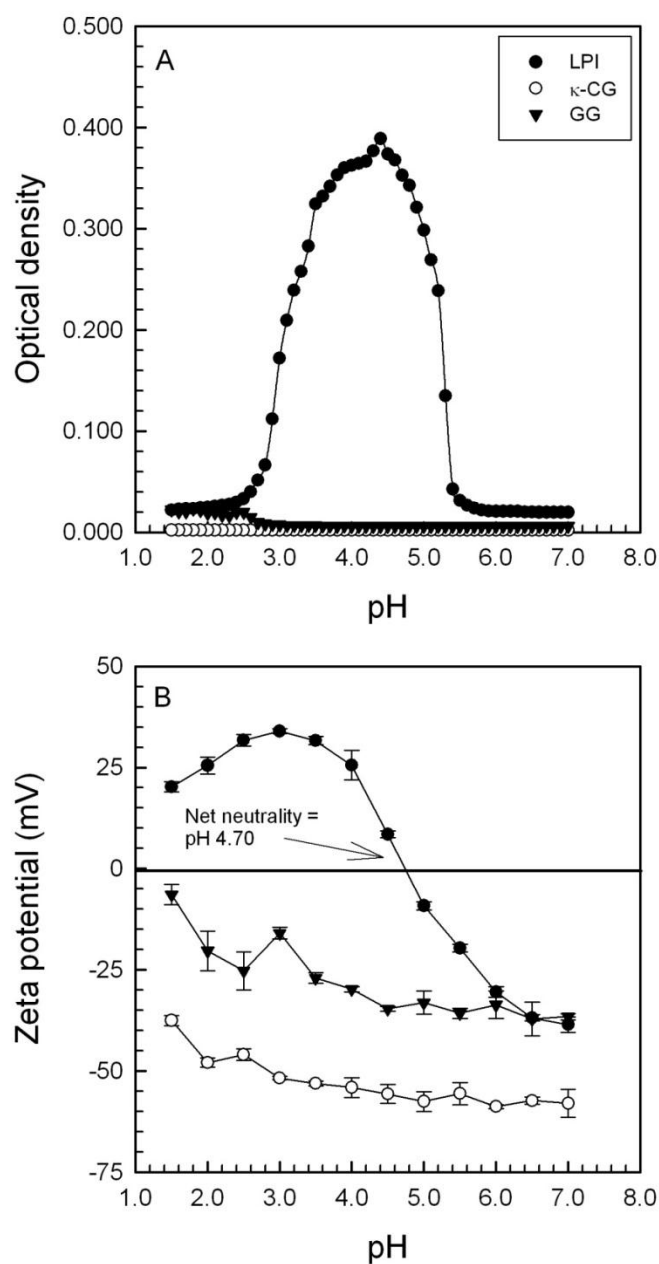


Figure 4.1 Mean (A) turbidity curves and (B) Zeta potential (mV) for homogenous (LPI, GG and κ -CG) as a function of pH at a total biopolymer concentration of 0.05% (w/w). Data represent the mean \pm standard deviation ($n = 3$).

Figure 4.2B shows the corresponding critical pH values associated with the formation of soluble and insoluble LPI: κ -CG complexes as a function of biopolymer mixing ratio. For the 1:1 LPI: κ -CG mixing ratio, reliable analytical results for pH_c and $pH_{\phi 1}$ were not obtained due to low

O.D. measurements. However, at the 5:1 LPI:κ-CG mixing ratio, soluble and insoluble complexes were found to occur at 6.12 and 4.30, respectively. As mixing ratios increased, pH_c and $pH_{\phi 1}$ shifted to higher pH ($p < 0.05$) until they tended to plateau as the biopolymer mixing ratios increased from 15:1 to 30:1 ($p > 0.05$). It was hypothesized that this trend was the result of a progressive rise in the number and size of LPI-LPI aggregates with increasing biopolymer mixing ratio, as the number of κ-CG chains (and subsequent electrostatic repulsive forces) were reduced until a critical LPI-LPI aggregate size was reached, after which a steady state was reached. It is believed that complexes were forming between small LPI-LPI aggregates and κ-CG chains, rather than individual protein molecules since the turbidity profile of the individual LPI solution (Figure 4.1A) was similar in the pH range with that of the mixed system. Similar trends describing the mixing ratio dependence of pH_c has been reported for canola protein-polysaccharide (alginate/ι-carrageenan) (Klassen et al., 2011), pea protein-GA (Liu et al., 2009), pea protein-alginate (Klemmer et al., 2012), and gelatin (type-A and -B)-agar (Singh et al., 2007) mixtures. Ratio dependence of $pH_{\phi 1}$, was thought to be due to a greater amount of protein molecules available per polysaccharide chain for binding with increasing biopolymer mixing ratio.

Similar mixing ratio- and pH-dependent turbidity profiles were observed for the LPI:GG system however O.D. values were higher because the suppression of LPI-LPI aggregation was not as substantial (Figure 4.3A) as that for the LPI:κ-CG system. Unlike with κ-CG present, turbidity profiles shifted slightly to lower pH, and the reduction in magnitude of O.D. relative to the individual LPI solution was much smaller (~difference of 0.100 O.D. vs. 0.250 O.D. with κ-CG). In addition, less mixing ratio dependence on the maximum O.D. was observed for this mixture. The critical pHs associated with the formation of soluble and insoluble complexes was also found to be less mixing ratio dependent than that observed for the LPI:κ-CG system (Figure 4.3B). These differences were believed to be due to the reactive sulphate group per disaccharide unit in the case of κ-CG, making it more electronegative than the carboxyl groups on GG's backbone and hence had a more pronounced effect LPI aggregation.

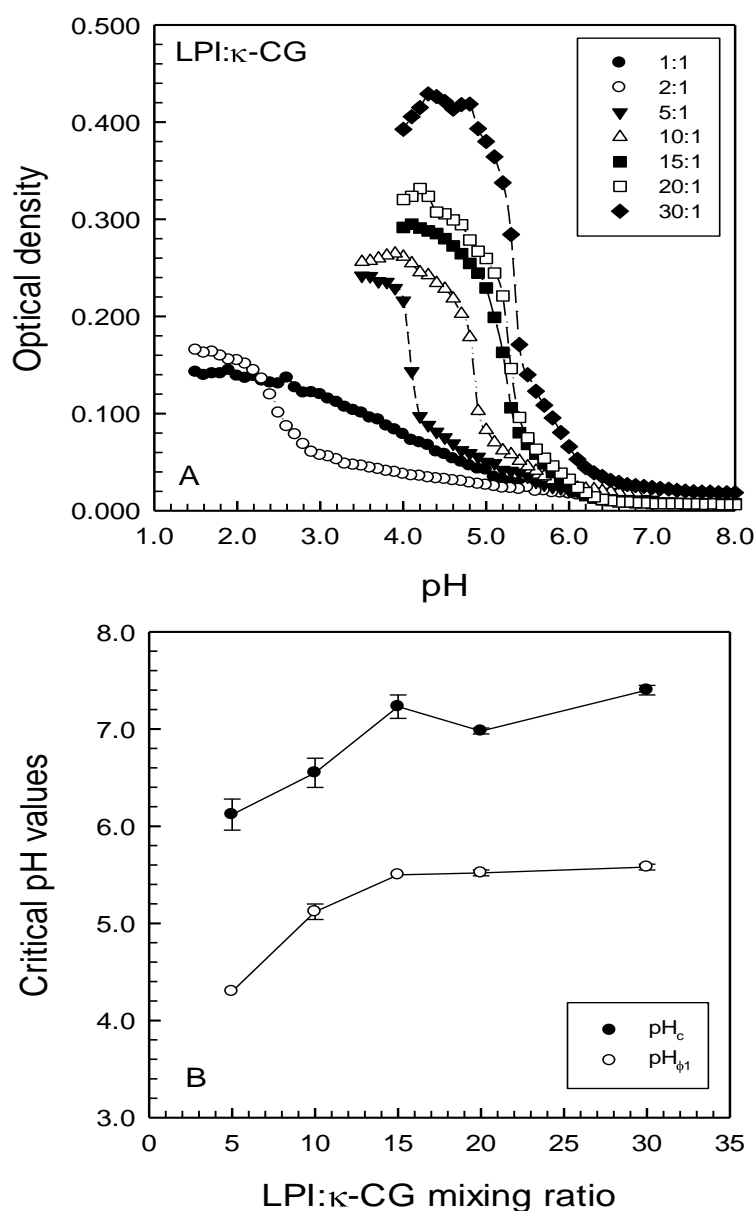


Figure 4.2 (A) Mean turbidity curves for LPI:κ-CG mixtures as a function of pH and biopolymer mixing ratio ($n = 3$). (B) Critical pH values associated with the formation of soluble (pH_c) and insoluble ($pH_{\phi 1}$) complexes for LPI:κ-CG mixtures as a function of biopolymer mixing ratios. Data represent the mean \pm standard deviation ($n = 3$). All samples were prepared to a total biopolymer concentration of 0.05% (w/w). Reliable estimates of pH_c and $pH_{\phi 1}$ could not be determined for ratios $< 5:1$ due to low O.D. readings. Data in (A) were removed from the profiles soon after O.D.(max), as precipitation ensured leading to unreliable O.D. data.

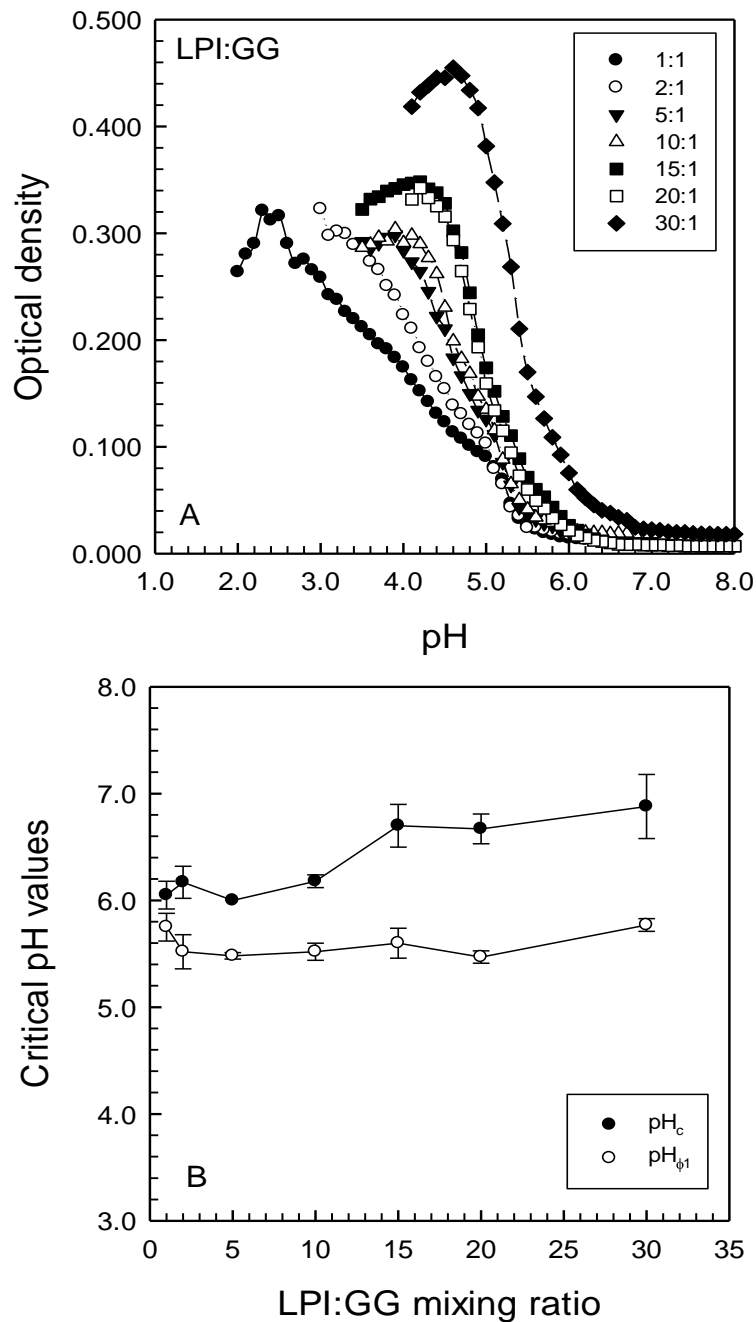


Figure 4.3 (A) Mean turbidity curves for LPI:GG mixtures as a function of pH and biopolymer mixing ratio ($n = 3$). (B) Critical pH values associated with the formation of soluble (pH_c) and insoluble ($pH_{\phi 1}$) complexes for LPI:GG mixtures as a function of biopolymer mixing ratios. All samples were prepared to a total biopolymer concentration of 0.05% (w/w). Data represent the mean \pm standard deviation ($n = 3$). Data in (A) were removed from the profiles soon after O.D.(max), as precipitation ensured leading to unreliable O.D. data.

For both LPI:κ-CG and LPI:GG mixtures, pH_c and $pH_{\phi 1}$ occurred at $pH > pI$ (4.70; Figure 4.1B) corresponding to conditions where biopolymers were of similar net charge, with the exception of $pH_{\phi 1}$ at the 5:1 mixing ratio. Complexation is believed to be initiated through the electrostatic attraction of anionic polysaccharides to positively charged patches on the protein's surface (Dickinson and Pawlowsky, 1998; de Vries et al., 2003; Weinbreck et al., 2004a). Complexation of both GG and κ-CG to LPI resulted in a shift in net neutrality of the formed complexes to a pH of ~4.36 for the 15:1 mixing ratio (Figure 4.4). Net neutrality occurred at pH values corresponding to the maximum of O.D. data for both LPI:κ-CG and LPI:GG mixtures. This phenomenon has previously been reported for whey protein-carrageenan (Weinbreck et al., 2004a), canola protein isolate-λ-carrageenan/alginate (Klassen et al., 2011), β-lactoglobulin-acacia gum (Schmitt et al., 1999), β-lactoglobulin-sodium alginate (Harnsilawat et al., 2006) and whey protein isolate- κ-/ι-/λ- carrageenan systems (Stone and Nickerson, 2012).

4.4.3 Effect of salts on the stability of electrostatic complexes at a 15:1 LPI: polysaccharide mixing ratio.

The effects of NaCl, KCl and CaCl₂ on the biopolymer complex stability (15:1 LPI: polysaccharide ratio) was investigated as a function of concentration (0-1500 mM) (Figure 4.5). In all cases, the addition of salts to individual LPI (Figure 4.5A), and mixtures of LPI:κ-CG (Figure 4.5B) and LPI:GG (Figure 4.5C) resulted in a reduction in O.D. with increasing concentration. Although κ-CG and GG are considered to be sensitive to K⁺ and Ca²⁺ ions, respectively, no salt-polysaccharide sensitivities were observed in this study. The reduction in O.D. for both monovalent ions was similar regardless of the biopolymer system tested, whereas the addition of a divalent ion resulted in larger reductions in O.D. than the monovalent salts for all systems and concentration used. The presence of ions was thought to shield the electric double layer on the surfaces of the biopolymers interfering with the complex coacervation process.

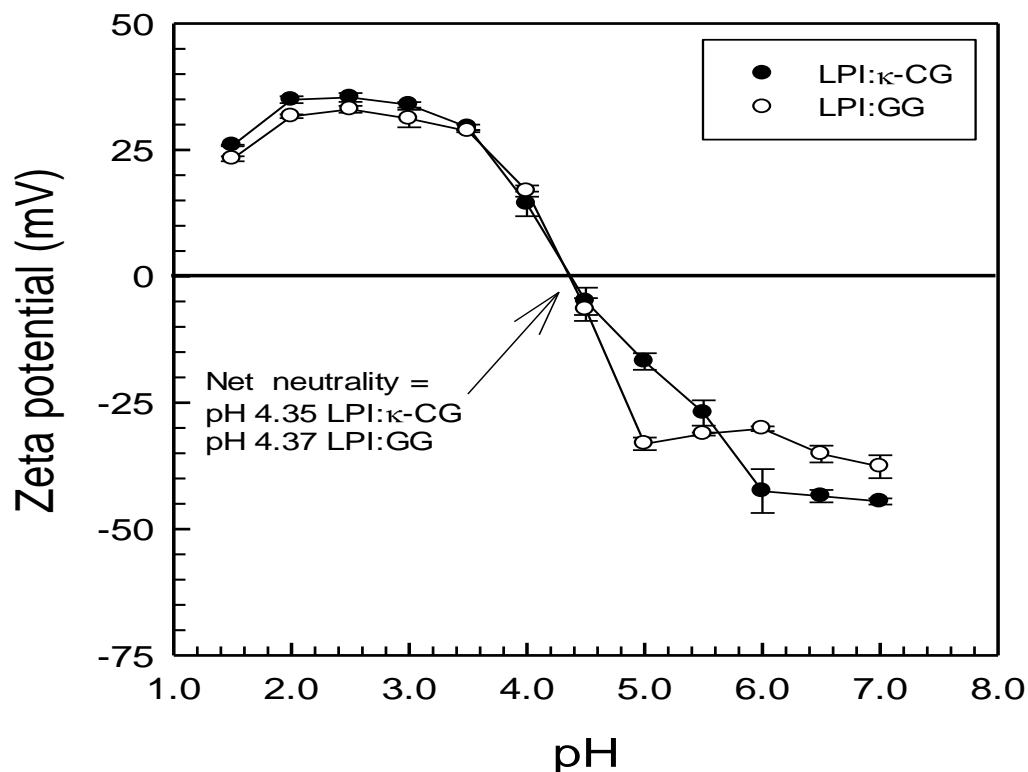


Figure 4.4 Zeta potential (mV) of mixed (LPI:κ-CG, LPI:GG; 15:1 mixing ratio) protein-polysaccharide systems as a function of pH. All samples were prepared to a total biopolymer concentration of 0.05% (w/w). Data represent the mean \pm standard deviation ($n = 3$).

4.5 Conclusions

Formation of electrostatic complexes within admixtures of LPI:κ-CG and LPI:GG were investigated as a function of pH and biopolymer mixing ratio. In all cases, complex formation occurred under pH conditions where biopolymers carried similar net charges presumed due to the strong electrostatic attraction between the highly negatively charged polysaccharides and positive patches on the protein's surface. Macroscopic phase separation occurred at pHs near the pI of LPI as evident by significant rise in O.D., followed by the precipitation of formed complexes at lower pH. Maximum LPI-polysaccharide interactions also seem to correspond to the 15:1 mixing ratio. The addition of salts acted to destabilize the electrostatic LPI-polysaccharide complexes through the shielding of charged reactive sites on the biopolymer's

surface. Although individual κ -CG and GG chains are considered to be highly sensitive to K^+ and Ca^{2+} , respectively, no polysaccharide-ion sensitivity was observed for the mixed systems.

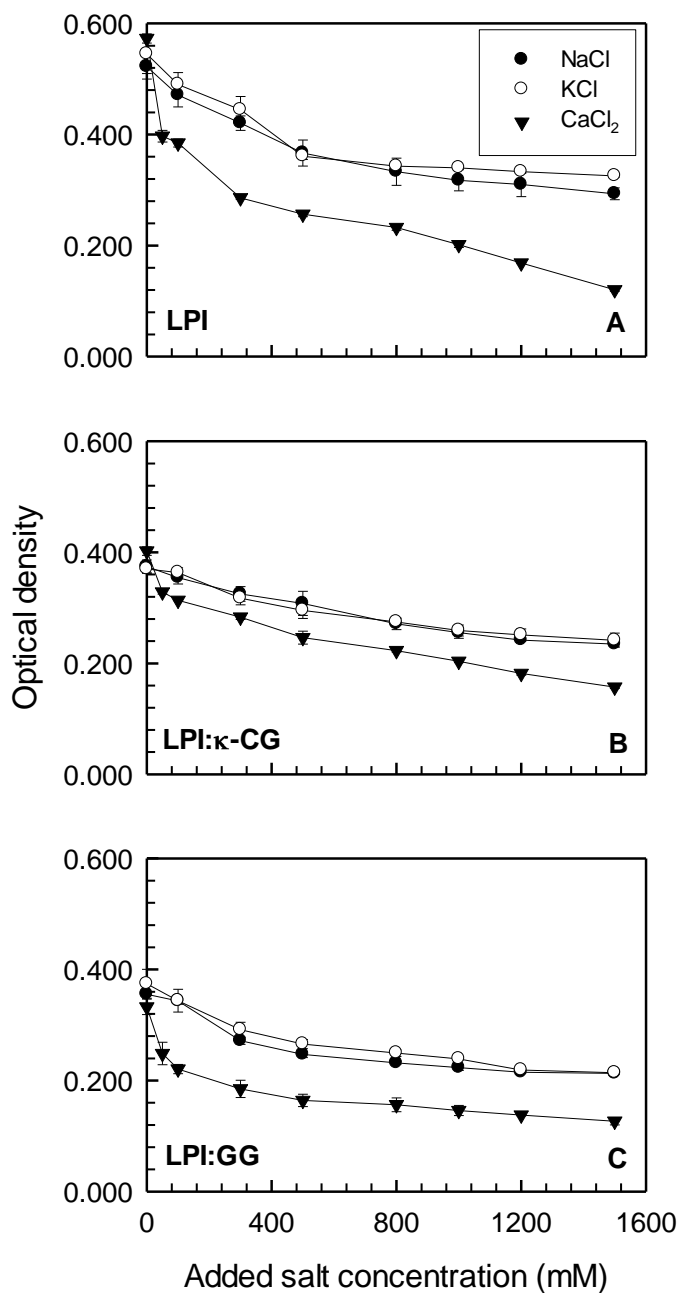


Figure 4.5 Mean effect of salt (NaCl, KCl, and CaCl₂) curves for O.D. of 15:1 biopolymer mixing ratio of LPI:GG and LPI:κ-CG mixtures at pH 4.37 and pH 4.35 respectively, as a function of salt concentrations (0–1500 mM). All samples were prepared to a total biopolymer concentration of 0.05% (w/w). Data represent the mean \pm standard deviation (n = 3).

5. GENERAL DISCUSSIONS

The overall goal of this research was to investigate mechanisms driving associative phase separation (or complex coacervation) involving a LPI and anionic polysaccharides. Specifically, the effect of pH, polysaccharide (κ -CG, GG and GA), biopolymer mixing ratio, ion type (Na^+ , K^+ , Ca^{2+} and urea), ion concentration, temperature and, presence and absence of the lentil seed coat, on the formation of soluble and insoluble complexes.

GA is considered to be a weakly-charged polysaccharide, whereas GG and κ -CG are both considered highly charged, despite both GA and GG having carboxyl groups as their reactive sites (de Kruif et al., 2004). GA is highly branched (providing shielding to reactive sites through steric hindrance) and hence has fewer exposed binding sites than GG, which in contrast is linear and has a higher charge density (one carboxyl group per tetrasaccharide repeating unit) (Nickerson et al., 2003; Dror et al., 2006). The polysaccharide κ -CG is considered to be more highly charged than GG, due to the presence of the more electronegative sulphate group per disaccharide repeating unit giving κ -CG a higher linear charge density. κ -CG and GG are widely used in the food industry due to their gel forming abilities, and interactions with K^+ and Ca^{2+} ions, respectively. GA on the other hand is employed as a thickening agent (Nickerson et al., 2003).

Typically, associative phase separation is based on electrostatic interactions between two oppositely charged biopolymers (e.g. protein + polysaccharide). During a turbidimetric pH acid titration, LPI switches from a net negative charge to a positive at its isoelectric point ($\text{pH} = 4.70$), whereas the anionic polysaccharides remains negative over the complete pH range examined ($>\text{pH } 2$). The formation of soluble complexes indicated by the first experimentally detectable rise in O.D. corresponding to initial interactions between LPI and the polysaccharide (Weinbreck et al., 2004a,b; Liu et al., 2009). In general, soluble complexes involving LPI and GA were found to occur at pH values where both biopolymers were of opposing net charge, whereas in the presence of GG or κ -CG complexation was initiated under solvent conditions where both biopolymers were of similar net charge. The latter was thought to be the result of an electrostatic

attraction between the anionic polysaccharide and positively charged patches on the LPI's surface.

As solution pH decreased, soluble complexes were thought to increase in both size and number until insoluble complexes were formed, as evident by large changes in O.D. and a transition from a transparent to a turbid sol. LPI:GA formed a typical coacervate-type complex which remained suspended. Complexation reached a maximum O.D. corresponding to where net neutrality occurred associated with a biopolymer electrical equivalent point. Similar behaviour has been reported for β -lactoglobulin-acacia gum (Schmitt et al., 2000), whey protein-GA (Weinbreck et al., 2003a), pea protein isolate-GA (Liu et al., 2009), soy protein-pectin (Jaramillo et al., 2011), gelatin-agar (Boral and Bohidar, 2010) and gelatin-chitosan (Gupta et al., 2007). As the pH was lowered further, O.D. declined gradually; indicating that, LPI:GA complexes were disassociating as the carboxyl site on the polysaccharide approached its pK_a . Similar to other plant protein-polysaccharide systems (e.g., pea protein isolate-GA (Liu et al., 2009)), a broad shoulder on the turbidity pH-profile was evident at pHs below pH_{opt} (max. O.D.), which is believed to be associated with protein-protein aggregate formation (Liu et al., 2009). In contrast, for LPI:GG and LPI: κ -CG systems, precipitates formed as solutions approached pH_{opt} . The precipitate structures are believed to be more compact than coacervate-type structures with stronger biopolymer interactions and less entrapped and mobile solvent (Boral and Bohidar, 2010).

Findings were comparable to other biopolymer mixtures involving weakly and strongly charged polysaccharides. For instance, coacervate-type structures have been found for whey protein-GA (Weinbreck, et al., 2003a) pea protein-GA (Chourpa et al., 2006; Liu et al., 2009), pea protein-chitosan (Elmer et al., 2011) and soy protein-pectin (Jaramillo et al., 2011), occurring over a narrow pH range initiated at pHs below the pI of the protein. In contrast, systems containing more highly charged polysaccharides, such as pea protein-alginate (Klemmer et al., 2012), and canola protein-alginate/ ι -carrageenan (Klassen et al., 2011) formed precipitate-type structures, often occurring at pH's above pI where both biopolymers have opposing net charges due to the attraction of positively charged patches on the protein's surface to the anionic polysaccharides.

Overall, as biopolymer mixing ratio was altered, so too were the turbidity profiles. Systems with higher levels of polysaccharides present were shifted towards lower pH conditions

due to a greater amount of electrostatic charge repulsion between neighbouring chains which was thought to inhibit protein-protein aggregation (Boral and Bohidar, 2010). The latter was thought to occur as a prerequisite to complex formation, due to overlapping turbidity-pH profiles between the mixed and homogenous protein solutions. Mixing ratios with higher amounts of protein resulted in less of a shift to acidic pHs than those with lower amounts, in some cases resembling that of the homogenous protein curve alone. Overall, biopolymer mixing ratio associated with the pH corresponding to the formation of soluble (pH_c) and insoluble complexes ($\text{pH}_{\phi 1}$) for all systems were found to rise (to higher pH) as mixing ratios increased up to a maximum pH and then plateaued. This plateau was thought to be associated with some upper critical size of protein aggregates being formed which then interacted with the polysaccharides within the mixture (Weinbreck et al., 2003a; Liu, et al., 2009).

The nature of interactions within the LPI:GA system was also explored in the presence of high levels of NaCl (100 mM), urea (100 mM) and elevated temperatures (60°C). Findings indicated that complexation was driven by electrostatic attractive forces between the two biopolymers, with secondary stabilization by hydrogen bonding. The role of hydrophobic interactions was thought to be associated with the stability of complexes (associated with the protein-protein aggregates involved with the complex) rather than complex formation. Similar findings were found for pea protein-gum Arabic (Liu et al., 2009) and canola protein-alginate/ κ -carrageenan (Klassen et al., 2011). Sodium chloride acted to screen or shield reactive sites on the protein and polysaccharide's surface to reduce the electric double layer of both biopolymers and interfering with the attraction between the two surfaces (Schmitt et al., 1999; Uruakpa and Arntfield, 2006b; Liu et al., 2009). Urea acted to break hydrogen bonds, whereas elevated temperatures acted to both break hydrogen bonds and promote hydrophobic interactions (Uruakpa and Arntfield, 2006b). Although the nature of interactions was only evaluated for the LPI:GA system, mixtures of LPI and GG/ κ -CG were also thought to follow the same mechanisms. For instance, the effect of increased levels of salt (NaCl, KCl and CaCl_2) on the stability of complexes formed between both LPI:GG and κ -CG were evaluated to find the dissolution of structures occurred (as evident by a reduction in O.D.) under all ionic conditions, with greater reduction occurring in the presence of CaCl_2 . The reduction in O.D. (or complexes) was thought to be similar as the nature of interaction study involving only 100 mM NaCl where the presence of excess co-ions and counter-ions resulted in shielding of reactive sites on both

biopolymers' surface. In contrast to the original hypothesis proposed, no salt sensitivities for LPI:GG- Ca^{2+} and LPI: κ -CG- K^{+} were observed. However, levels of both polysaccharides were sufficiently low, that may have prevented detection of these sensitivities. Conditions where biopolymer concentrations were raised may produce different results than reported in this study.

The seed coat primarily is comprised of fibre, polyphenols or tannins and other anti-nutritional factors (Wang, 2005). The minor effect on complexation is presumed to be related to increased hydrophobic interactions which only helps in stabilizing the complexes but not in their formation; or due to the removal of polyphenols, tannins, fibre or other nutritional factors that could influence protein aggregation.

6. GENERAL CONCLUSIONS

Overall, complexation of LPI with both weakly (GA) and highly (κ -CG, GG) charged polysaccharides behaved similarly to other legume protein-polysaccharide mixtures indicating a similar mechanism is driving complex formation. Complex formation followed two structure forming events associated with the formation of soluble and insoluble complexes with initial interactions occurring slightly above the pI of the LPI where all biopolymers carried similar net negative charges. Mixed systems also showed similar pH and mixing ratio-dependence as animal protein-polysaccharide mixtures such as, xanthan gum-gelatin (Lii et al., 2002), β -lactoglobulin-pectin (Girard et al., 2002) and whey protein isolate- ι - κ - λ -carrageenan (Stone and Nickerson, 2012), with a few exceptions: These include: a) the mixing ratio dependence of pH_c (soluble complex formation) which tends to be relatively independent of mixing ratio rather than increasing up to a plateau as found in this study; and b) the presence of a broadened shoulder at $\text{pHs} < \text{pH}_{\text{opt}}$. Both of these exceptions are believed to be associated the presence of smaller protein aggregates especially when the mixtures include milk proteins.

LPI:GA formed a typical coacervate-type complex, whereas LPI:GG and LPI: κ -CG systems formed precipitate-type structures. All systems were found to be highly mixing ratio dependent with interactions occurring over a narrow pH range. The nature of protein-polysaccharide interactions was found to be primarily driven by electrostatic attractive forces with secondary stabilization by hydrogen bonding. Hydrophobic interactions were thought to play a role in stabilization of protein-protein aggregates involved within the complexes, rather than contribute to its formation. Removal of the seed coat (dehulling) had only a minor effect on complex formation and no polysaccharide-ion sensitivities were observed for the mixed systems tested (i.e. LPI:GG- Ca^{2+} and LPI: κ -CG- K^+).

7. FUTURE STUDIES

As market trends continue to shift towards plant proteins and away from animal-derived ingredients, a greater understanding of protein-polysaccharide interactions and their phase behaviour in foods is needed in order to better control their effects on food quality, structure and texture. A greater understanding of structure-dynamic-function relationships will hopefully improve the use of plant protein ingredients in a wider range of food and non-food applications. The latter may include, films/packaging, controlled delivery systems (nanoparticles, microcapsules, capsules), new separation technologies and so on.

Despite many studies (Liu et al., 2009, 2010a,b; Klassen et al., 2011; Jaramillo et al., 2011; Klemmer et al., 2012) looking at solvent effects (e.g., salts, temperature and pH) and biopolymer characteristics (e.g., mixing ratio and polysaccharide-type) on complexation involving plant proteins, very little is still known about the underlining mechanism and formed structures. For instance, the effect of biopolymer size, degree of branching, chain stiffness, total biopolymer concentration and aggregation on the formation of soluble and insoluble complexes remains relatively unknown. Protein aggregation prior to complexation could have a large impact on the size and stability of formed complexes. Molecular weight of proteins (or polysaccharides), degree of branching and relative chain stiffness can all impact the spatial arrangement of reactive groups (and chains) needed to form electrostatic interactions between biopolymers, and could impact the solubility of formed structures (e.g., instability via bridge flocculation, i.e., a single polysaccharide may complex with multiple proteins forming larger flocs with reduced ability to remain suspended in solution). Furthermore, the role of individual proteins (e.g., legumin vs. vicilin) on complexation with polysaccharides remains unknown, and their contribution to the coacervation behaviour of the mixed isolate. Complexation studies testing these parameters could involve similar turbidimetric analyses as performed in the current research, or other light scattering and imaging techniques (e.g., confocal scanning laser microscopy); except with greater consideration to the biopolymers used or pre-treatment conditions (e.g., enzymatic hydrolysis, cross-linking).

The role that complexation-induced conformational changes have on protein structure and also on complex stability and/or functionality is also poorly understood. Depending on the strength of interactions, biopolymers involved and solvent conditions, varying levels of conformational change to the protein could ensue, leading the exposure of buried hydrophobic and/or hydrophilic groups. This change could have a negative, neutral or positive effect on its surface chemistry and its ability to align at the air-water or oil-water interface in the case of foams and emulsions, respectively. The level of conformational changes, and ensuing protein-protein and/or complex aggregation may also influence whether coacervate or precipitates form. Conformational changes to the protein structure during complexation could be measured using fluorometry, circular dichroism or Raman spectroscopy.

The morphology of a coacervate or precipitate structure is also relatively unknown. The former has a more open structure with a lot more fluidity of biopolymers and mobility of entrapped solvent, allowing it to remain suspended in solution (within a coacervate-phase). In contrast, precipitate structures tend to be more compact, with greater biopolymer rigidity and less entrapped solvent present. Morphological analyses by confocal scanning laser microscopy, atomic force microscopy and/or electron microscopy of coacervate or precipitate phases may - give greater insight to biopolymer ordering within these structures. Such knowledge could then be useful in terms of tailoring structure of biomaterials and/or for controlled release purposes.

Furthermore, as polysaccharides complex with proteins, surface chemistry of the plant protein changes altering its functionality in a negative, neutral or positive way. Comprehensive studies on the functionality of formed complexes relative to the biopolymers alone should be performed using literature tests for solubility, emulsification, foaming and water/oil holding capacities in simple systems for product development purposes. Along with more in-depth rheological studies on their thickening and gelling properties, or influence on the stability/in-stability of multi-phase systems (e.g., foams and emulsions). In terms of food systems, ingredient interactions (e.g., sugars, lipids) and processing (e.g., stirring, whipping, pumping or other forms of shear) become significant factors influencing phase behaviour, which could then influence food structure and quality. Of particular interest is the lack of O.D. in the mixed systems at pHs <4.0 for the mixed systems of 1:1 and 2:1 LPI: κ -CG/GG. In contrast, O.D. for LPI alone indicated high O.D. at pH 4.0. Further studies are needed to better understand the effects of the

addition of polysaccharides at these ratios on the solubility of LPI. Findings could lead to a means of keeping LPI in solution for more acidic applications.

Lentil proteins, like other plant proteins are advantageous due to their relatively low cost (relative to animal proteins), nutritional value and functional attributes to make them attractive ingredients to replace animal-based alternatives. However, a greater understanding of performance and ingredient interactions is needed for more wide spread integration to food and non-food applications.

8. REFERENCES

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